Optimisation of Solid-State Fermentation for Enzyme Production

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Abstract

Optimisation of the solid-state fermentation process was carried out using *Aspergillus niger* and *Rhizopus oligosporus* for the production of phytase and protease enzymes. Experimentation was conducted using koji flasks in the laboratory, in addition to production scale koji tray experiments in industrial scale incubation chambers. Heat removal from the chambers was carried out through the use of water sprays and aeration systems, with water removing 65-85% of the heat. Process optimisation was carried out using quadratic optimisation experiments to ascertain the optima for temperature, pH, moisture and mineral supplementation. Findings included improvement of yield through both process standardisation and parameter optimisation. The methodology reduced the time and repetitions required for successful process improvement. Supplementation of the wheat bran medium, with a yeast extract product or with mineral supplementation, resulted in more consistent and higher yields of phytase activity. Higher enzyme yields would lead to reduced product costs and allow for more widespread use of the product in animal feed. This could impact the energy, feed, and food industries simultaneously and there may be potential to integrate these three industries through this technology.

Key Words: Aspergillus, industrial scale koji, optimisation, phytase, protease, Rhizopus, solid-state fermentation, wheat bran

Dedication

To my incredible parents

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1.1.1 What are enzymes?

Throughout time, microorganisms have fermented materials in order to break them down into components useful to themselves and the environment around them, and thus were able to survive and multiply. Through evolution, these microorganisms diversified into thousands of species with a vast array of capabilities and characteristics. All of these organisms use enzymes to carry out their important work.

Enzymes are critical to life and the breakdown of nutrients for all living things. They are naturally occurring catalysts, produced by all living organisms that speed up reactions. Between 3,000 and 4,000 different enzymes have been identified and most are proteins (Sheppy, 2001). Enzymes function by temporarily binding to one or more reactants in a reaction, and lowering the activation energy required for such a reaction. Enzymes are normally made up of amino acids, which create a three dimensional structure in which the amino acids responsible for the catalysis process are positioned close to reaction sites. Because of this structure, they are sensitive to the conditions in which they function, particularly parameters such as pH and temperature.

1.1.2 A short history of human use of enzymes

Enzymes have been used by humans for some time, initially without their knowledge. Their use was mainly focused around food preparation. For more than 10,000 years fermented food production of beer, wine, bread, and cheese is believed to have been practiced. This connection between fermentation and enzymes is what made up the entomology of the word, enzyme, which comes from the Greek for 'in yeast' (en-zyme). Microorganisms in these preparations are primarily responsible for converting carbohydrates to carbon dioxide, ethanol, and lactic acid. Cheese making, soy sauce

production, baking, and brewing all incorporate enzymes and have done so, well before microbiology and enzymology were understood. Sometimes, microorganisms fermented the materials and created their own enzymes and other times enzymes were added in some manner. Examples of this addition can be seen in enzyme rich malt addition in brewing and in the use of protease-full papaya leaves in some parts of the world for tenderising during meat preparation. It is interesting to note that researchers in the areas of food preparation and fermentation opened up the area of enzymology (Amado, 1993).

The history of industrial enzymes dates back to the isolation of diastase (amylase) from a malt extract by ethanol precipitation in 1833, the proteolytic enzyme pepsin isolated from a pig's stomach in 1850 (Amado, 1993), and to 1874 when Hansen manufactured chymosin from the stomach of calves for the manufacture of cheese. Jokichi Takamine in 1894 was the first person to isolate an enzyme from a microbial source when he isolated taka-diastase from Aspergillus as a digestive enzyme (Fig. 1.1). From there an industry grew that now is based significantly on genetic modification and biotechnology.

Figure 1.1 Jokichi Takamine - the 'father' of the Solid-State Fermentation (SSF) process for biotechnological uses.

Today the enzyme industry is an over two billion dollar industry. A survey, conducted in 2003, concluded that food, when grouped together, was still the largest user of enzymes (~40%), broken down into baking (11%), starch (10%), brewing (9%), dairy (5%), fruit and juice (4%), and flavours (2%). Over 27% of industrial enzymes are sold into the detergent industry. Textile and paper manufacturing makes up 4-6%, and the leather industry makes up 1% of the market (see Fig. 1.2). Animal feed is one of the fastest growing areas, having increased from 1% to 9% of the total business in fifteen years (Novozymes Annual Report, 2006). Due to the recent escalation in ethanol production in the US, these percentages are starting to change dramatically.

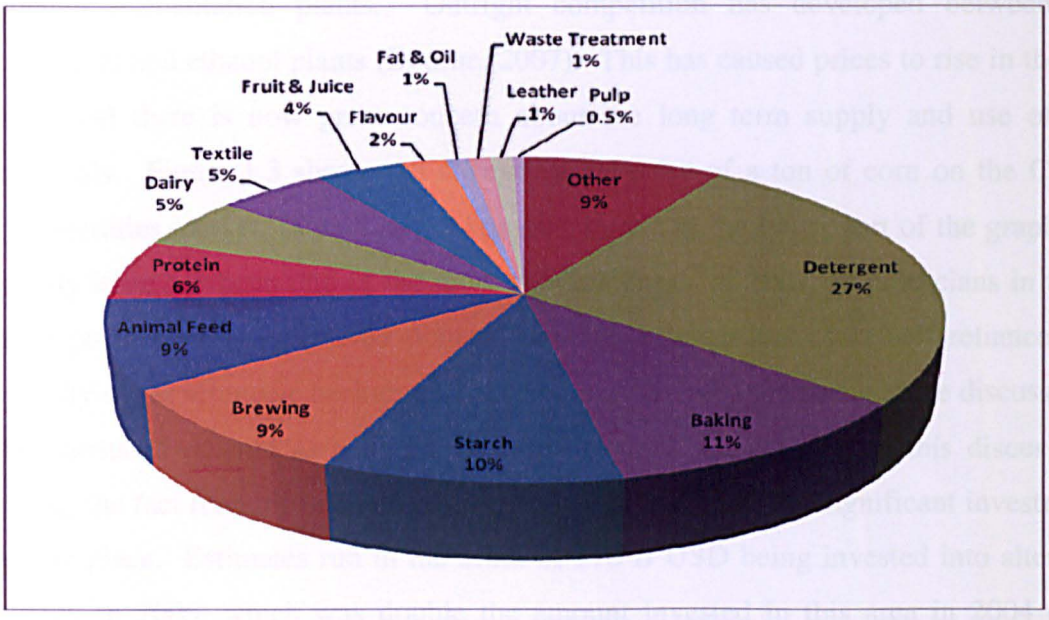


Figure 1.2 Percentage breakdown of global enzyme industry.

As the industry moves forward, genetic engineering continues to be at centre stage. New products in the areas of bioethanol are currently a primary focus. The conversion of grains and fibrous materials into cellulosic ethanol is an exciting opportunity for the use of genetically manipulated organisms (GMO). However, other techniques for enzyme production are starting to be investigated as criticism by environmentalists steers some to look at greener technologies. Several niche market players are looking to this technology to gain commercially while satisfying consumer demands.

The global community has also awoken to the reality that climate change may be a reality here to stay and that choices must be made to curb energy usage and greenhouse gas emissions. It is estimated that the temperature of the globe will increase 3.7 to 9°C in the next 50 years based on the decisions made today on energy choices (McKibben, 2007). This has also been sparked by higher energy prices. Due to these changes in prices and availability, mixed with the changing environmental concerns and choices, grains have rapidly and suddenly been put under strain to meet human demands for the production of food, feed, and fuel. These grains, predominately corn, traditionally would have been used just for animal feed and human food. Now, through the production of ethanol for fuel, seen by many as the most realistic of the potentially environmental friendly options for energy production, grains are being consumed by ethanol fermentation plants. Outright competition has developed between meat producers and ethanol plants (Bourne, 2007). This has caused prices to rise in the short term and there is now great concern about the long term supply and use of these materials. Figure 1.3 shows the change in the price of a ton of corn on the Chicago commodities market, as well as the volumes traded in the lower part of the graph. The supply issue has been one of the most political issues of 2007, as politicians in the US have pushed for an improved domestic energy balance and more self reliance. The validity of the scientific background of this issue has been the focus of the discussion, as the merits of ethanol as a liquid fuel are debated. Despite where this discussion is going, the fact remains that new plants are being built and very significant investment is taking place. Estimates run in the order of $70 B USD being invested into alternative energy in 2006, which was double the amount invested in this area in 2004 (Barta, 2007). This would make this effort the biggest construction and investment programme ever to take place in agriculture in the United States (Personal communication, Bob Dinneen, Renewable Energy, 2007).

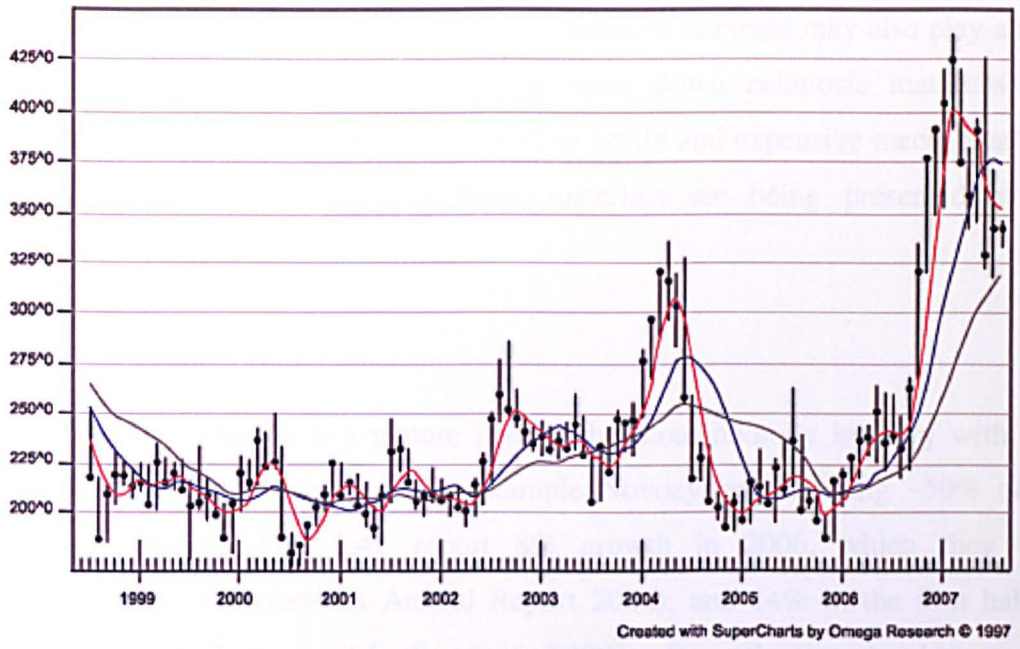


Figure 1.3 Monthly corn price (TFC commodity, 2007).

The pressure on grains will mean that stocks will start to drop and consumers will have to become flexible or else pay a premium for what they use today.

The use of traditional and novel enzymes could significantly impact this situation. If alternative raw materials could be utilised for animal feed or energy, the pressure on corn prices would decrease. As the likely candidate for the most price sensitive of the three industries, animal feed is already taking the lead in terms of looking for alternatives. Products such as cassava, palm kernel meal, wheat bran, peanut hulls, rice hulls, corn stover, and corn cobs are now being included in animal feed diets, when previously they would not have made up significant portions of animal diets. Also, traditionally used products such as Distiller's Dried Grain's with Solubles (DDGS) are being reviewed in order to investigate whether higher levels of inclusion could be a reality. This is occurring behind a backdrop of higher scrutiny by consumers into knowing what is in their food and their animal's food (Economist, 2007). Enzymes will play a central role in this discussion and will likely be the answer to the utilisation of both non-traditional materials and the increase in the inclusion rate of fibrous traditional materials.

The energy sector is not far behind and alternative enzymes may also play a significant role in this area. Enzymes that can break down cellulosic materials will save tremendous amounts of energy by avoiding costly and expensive mechanical processes and physical modifications of these materials are being presented as cellulose conversion methods.

The enzyme industry is a mature part of the biotechnology industry with an annual growth rate above average. For example Novozymes, claiming ~50% of the total enzyme market (Fig. 1.4), report 8% growth in 2006, which they find only "satisfactory" (Novozymes Annual Report 2006), and 14% in the first half of 2007 (Group financial statement for first half of 2007). Recently, there has been considerable pricing pressure from newly emerging biotechnology companies, producing cost-efficient enzyme concentrates with lower research and development expenses, compared to Novozymes and Genencor, the two market leaders.

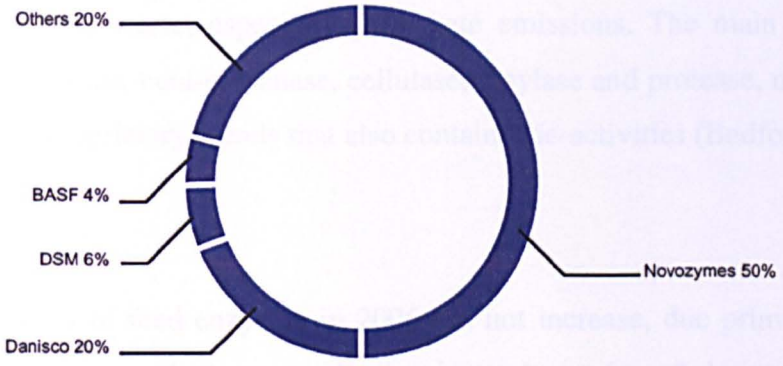


Figure 1.4 State of competition with enzymes for industrial use.

Novozymes reported sales of $1.17 billion in 2006, and its market share was 50 – 55%. This brings the total 2006 market to $2.1 – 2.3 billion and growing by 10% for 2007.

1.1.3 Use of enzymes in the feed industry

During the second half of the twentieth century, there was a significant increase in the

levels of cereals used in animal feed. This was motivated by economic pressure pushing the cost of animal feed lower. For example, between 1972 and 1992 the main source of protein in poultry diets changed from fishmeal to soybean (Rumsey, 1993). With this change new challenges arose, such as the removal of anti-nutritional factors and the development of mineral and amino acid supplements, to make up for the reduction of these nutrients when moving to these cereal based diets (Mullaney *et al.*, 2000). Over this time, the animals also experienced a large increase in size and production. In order to overcome this change, great focus was put on the efficiency of the utilisation of the materials in diets. Enzymes were a near perfect way to obtain more out of what was already there and research into this area has increased significantly over the past twenty years (Pierce, 1999).

1.1.3.1 Commonly used enzymes

In animal feed, the use of enzymes expands the range of feed ingredients, assists with the degradation of anti-nutritional factors (ANF) (these cannot be used by the animal and may absorb nutrients), improves feed conversion and weight gain and reduces animal waste, especially phosphate emissions. The main feed enzymes are phytase, xylanase, beta-glucanase, cellulase, amylase and protease, marketed as pure enzymes or as proprietary blends that also contain side-activities (Bedford and Apajalahti, 2001).

Sales of feed enzymes in 2006 did not increase, due primarily to lower prices for the phytase product group, changes in product mix and due to emerging local competition in China. Novozymes anticipates growth in sales of feed enzymes of 1-3% in 2007 (Novozymes Annual Report, 2006).

With the global market of feed enzymes of $250 million, average enzyme cost of $2/ton of feed and global feed production of 600 million tons, only 20% of world feed is treated with exogenous (microbial) enzymes. Diets, based on small grains (wheat, wheat bran, triticale, barley, rye, palm kernel meal) are treated almost at 100%. Diets based on grains with low non-starch polysaccharides (NSP), for example maize, sorghum, rice or

soybean meal, are treated to a much lower extent, and mostly with phytase and protease, which provide benefits in addition to the degradation of the non-starch polysaccharides. The poultry feed market is penetrated at a higher percentage than the pig feed and aquaculture feed market, and the ruminant feed market to date is almost untouched, partially because of high fragmentation.

The main profit in enzymes for animal feed is derived from providing nutritional solutions (about 70%), while the value of enzyme concentrates amounts to only 30%.

In the feed industry, enzymes have been used for quite some time, with and without the knowledge of the farmer, for ruminant and non-ruminant animals. Initially most of this enzyme use was haphazard and not very well understood scientifically. Traditionally non-specific fermentation products were used. Over the past twenty years, the commercial application of enzymes as a feed additive has become an accepted idea. During this time, the feed enzyme industry emerged and since then the industry has gone through several phases of development. Enzymes used in ruminant diets tend to focus on cellulase and hemicellulase activities, due to the high levels of cellulose and hemicelluloses in plants that make up ruminant diets (Beauchemin *et al.*, 2003). The objective is to increase this metabolism in the rumen in order to enhance growth of the animal and/or milk production.

In non-ruminant diets, there is a more significant focus on anti-nutritional factors that cannot be broken down by the animal. The initial focus was to use enzymes to enhance nutrient digestibility, primarily focusing on removing the anti-nutritive effects of non-starch polysaccharides, such as arabinoxylans and β-glucans, from broiler diets based on viscous grains such as wheat, rye, barley or triticale. Exogenous enzyme addition can decrease the percentage of the total feed that these anti-nutritional factors encompass and turn this negative into a positive by releasing energy when the anti-nutritional factors are broken down (Bedford and Schulz, 1998). In the early 1990s, there was a significant change as the scope of enzyme applications expanded to consider nutrients

other than the non-starch polysaccharides and benefits other than digestibility enhancement. Phytase, which will be discussed later, is a good example of this change.

The next step in the industry was for enzymes to be used in non-viscous grain diets, such as sorghum and corn. Although such a use is not uncommon in some parts of the world, the industry is still in search for highly efficacious enzymes for non-viscous cereal grains. Subsequently, the application of enzymes to non-cereal grain components of the diet provides a great potential for improvement in the efficiency of these animal feeding systems. These vegetable protein sources are often high in non-starch polysaccharides, which are poorly characterised in regard to their molecular structures. Significant progress has been made on characterisation of the non-starch polysaccharides in soybeans, but improvements in the ability to consistently improve the digestibility of vegetable proteins, through the use of enzymes, is still sought along with acceptance by the market of these solutions.

Some recent data demonstrates the role of glycanases (carbohydrate degrading enzymes) as an alternative to in-feed antibiotics (Choct, 2006). It is possible to produce enzymes tailored for specific low molecular weight carbohydrates *in vivo* to improve health status, de-activation of anti-nutrients starting with non-starch polysaccharides and phytate and now extending further, to the degradation of non-conventional feed resources to create more available energy (Choct, 2006). Swine are often unable to utilise 15-25% of their diets (Sheppy, 2001). This is in front of a backdrop of an industry where feed comprises 55% of total costs for animal production, and all costs are rising (Lyons, 2007). The economic opportunities to decrease the amount of under utilised material in the diet are quite clear.

Equally, due to increasing corn prices (Fig. 1.3) and other traditional raw materials, there is a great interest in using less expensive by-products. These by-products tend to be less readily digestible and therefore of less nutritional value. Utilisation of enzymes to make them more digestible will increase their nutritional value to the animal. Often

materials that cannot be digested by endogenous enzymes are also trapping valuable proteins, minerals, and starches that the animal could utilise. Young animals also benefit from exogenous enzyme addition, as their enzyme production systems may not be fully developed (Sheppy, 2001). Today use of enzymes in the feed industry is widespread, with pure enzymes, nonbacterial direct-fed microbials, and specifically fermentation products tailor-designed for their enzyme applications.

1.1.4 Phytase case study

One of the biggest successes in animal feed applications for the enzyme industry has been the commercialisation of the phytase enzyme. The sales of phytase once exceeded $500M per year (Abelson, 1999), although prices have lowered significantly reducing this market value, despite widespread demand. One of the most important aspects of this success is that this is the only enzyme specifically developed for and by the animal feed industry.

Phosphorus (P) is known to be critical to many biological functions. It is second only to calcium (Ca) in body mineral concentration. The vast majority of phosphorus (~80%) is located in the bone matrix due to its vital role in the development and maintenance of skeletal tissue (Simons *et al.*, 1990). The other 20% is located in the soft tissues and performs biochemical functions such as being part of nucleic acids (Coon and Manangi, 2007) and therefore the backbone of DNA, ATP formation (energy transfer), functioning as the endocrine's second messenger, and being a component of phospholipids (Pierce, 1999).

In nature, phosphorus is stored in many forms, with phytate, which is the salt of phytic acid, being one of them. Substantial quantities of phytic acid occur naturally in plants (Jongbloed and Lenis, 1998), roughly at a level of over two thirds of the total phosphorus in the plant material tissue (Simons *et al.*, 1990). Phytate is accumulated during the ripening process of a plant (Asada *et al.*, 1969). It is normally found in the

bran in small grains (aleurone layer, testa, and pericarp), in the germ in grains such as corn or maize, throughout the protein complexes in soybeans, and in legume seeds in the cotyledon (Baker *et al.*, 2001). It is thought that phytate also functions as an antioxidant in seeds and has a very high chelating potential. This is shown in the case of iron, most of which is complexed with phytate in plant seeds, eliminating the potentially lethal combination of free iron and unsaturated fatty acids together in the seed, thus protecting the plant (Mullaney *et al.*, 2000). This chelation also affects other minerals such as calcium, zinc, copper, cobalt, manganese, and magnesium (Kornegay, 2001).

As economic pressure increased on the animal feed industry, there was a shift towards less expensive, cereal based diets, both in smaller and larger animals (Mullaney, *et al.*, 2000). When these plants are used in monogastric diets, this phytic acid is consumed by the animal and is not effectively digested (Torre *et al.*, 1991). Therefore easily available sources of phosphorus must be added to these diets in order for the animals to receive a sufficient supply. These sources were often selected based only on price. Inexpensive rock phosphate (calcium phosphate) was often used. The result is that the animal utilises the supplemented phosphorus and the unusable phytin phosphorus is excreted in the animal's waste and thus into the soil. However, unfortunately these levels turned out to be much higher than the land could absorb (Mullaney *et al.*, 2000). This phosphorus does not leach through the soil but is absorbed into soil particles and enters waterways, such as rivers and oceans, when soil erodes (Loer, 1984). Phosphorus is typically the most limiting nutrient in aquatic plant growth (Pierce, 1999), and thus this is of grave environmental concern. Upon entering the water, this topsoil which is rich in phosphorus can lead to algal blooms, which use up oxygen thus unbalancing the ecosystem, which ultimately can lead to diminishing fish populations. Phosphorus and phytic acid pollution can also impact other ecosystems as well as impacting protein and mineral digestion in the animal (Maenz, 2001). By 1991, it was estimated that 15.5 million tons of dry swine manure containing 460,000 tons of phosphorus were excreted annually in the US (Sweeten, 1991). Today this number would be higher due to the increase in animal production, especially in areas such as Arizona and New Mexico.

These environmental concerns have motivated researchers to look for solutions to this problem and also to reverse this situation by creating energy out of the phytic acid and at the same time decreasing the potential ecological impact. In the Netherlands, where there is a nitrogen, potassium, and phosphorus imbalance and 80% of the increased accumulated mineral excess comes from animals (de Boer, *et al.*, 1997), laws have been enacted to limit the amount of phosphorus that can be spread on land and other governments have followed their lead (Jongbloed and Lenis, 1992).

Endogenous phytase is present in ruminants due to the microorganisms in the rumen, but this is not the case in non-ruminants. Phytase was initially discovered by Suzuki in 1907 (Mullaney, *et al.*, 2000). Early steps into the commercialisation of this enzyme took place in 1968, when the International Mineral and Chemical Company, a supplier of rock phosphate to the animal feed industry, looked strategically ahead at their future business, and sponsored research that ultimately led to the identification of an *Aspergillus niger (ficuum)* strain that expressed significant levels of phytase activity. The levels were not high enough for this to be interesting commercially and therefore the project was halted (Mullaney, *et al.*, 2000). The first test involving exogenous microbial phytase addition to feed probably took place in the early 1970's and showed an increase in the availability of phosphorus in chicks fed a diet of soybean and maize along with an Aspergillus preparation (Coon and Manangi, 2007). At that stage this was considered to be too costly and political pressure regarding environmental impact was not yet an issue. As that pressure increased towards the end of the 1980's, with the Dutch mandate on pig farmers, technology in the form of genetic modification was also being investigated in the search for an economical solution. A reduction of phosphorus excretion between 20-50% was developed (Kornegay, 2001) and since that time several more phytase products have been introduced into the market. These products are from both genetically modified and non-genetically modified sources. A decrease in the amount of phosphorus that is excreted from the animal, and therefore reduction of a potential pollution problem, has been achieved. Phytase has also been shown to lower feed costs, as the phosphorus present in the plant material is better utilised and less phosphorus has to be added to the diet (Coon and Manangi, 2007).

It was later found that one of the other major benefits of the enzyme was its impact on minerals and nutrients, other than phosphorus. In both poultry and pig diets, calcium, zinc, protein/amino acid and energy availability were seen to increase when phytase was used at adequate rates. This is because many of these nutrients are chelated as previously mentioned with phytate and are not available until the phytate is broken down by the phytase enzyme. Particularly for monogastrics, this renders many of these minerals, along with the phosphorus, inaccessible (Kornegay, 2001).

Two classes of phytase are recognised, based on the position of the first phosphate hydrolysed from phytate (myo-inositol-1,2,3,4,5,6 hexakis dihydrogen phosphate), namely 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.26). The 3-phytase class is mainly of microbial origin, whereas the 6-phytase class is derived from plants.

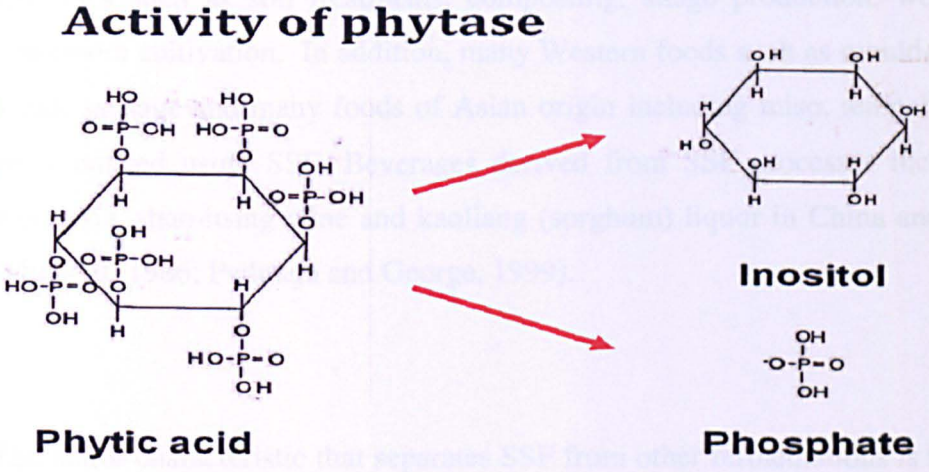


Figure 1.5 Activity of phytase enzyme.

As illustrated in Figure 1.5, phytase breaks down phytic acid into inositol and releases phosphorus for the animal to utilise. Phytic acid consists of a sugar (similar to glucose) called myo-inositol, to which the phosphate (PO_4) groups are covalently linked. Phytase releases these phosphates from the inositol ring. Phytase is mixed with the feed and the phytate is digested in the gut of the animal, providing essential phosphate for the animal.

Although different strains of bacteria, yeast and fungi have been used for the production of phytase, in both submerged and SSF systems, strains of *A. niger, A. sporolus* and *A. ficuum* are the most common organisms used for the commercial production of phytase (Pandey *et al.*, 2001b). By far, most phytase to date has been produced through conventional submerged fermentation but there are other ways to produce phytase along with other enzymes of interest to the feed industry, through techniques such as SSF.

1.2 SSF and the Koji System

1.2.1 What is solid-state fermentation (a basic definition)?

The origin of SSF can be traced all the way back to bread-making in ancient Egypt, over 4000 years ago. Solid substrate fermentations also include several well known microbial processes such as soil treatments, composting, silage production, wood rotting and mushroom cultivation. In addition, many Western foods such as mould-ripened cheese, bread, sausage and many foods of Asian origin including miso, tempeh and soy sauce are produced using SSF. Beverages derived from SSF processes include ontjom in Indonesia, shao-hsing wine and kaoliang (sorghum) liquor in China and saké in Japan (Mudgett, 1986; Padmaja and George, 1999).

The major characteristic that separates SSF from other fermentations is the low level of moisture at which the fermentation takes places. SSF is generally defined as a fermentation which is carried out on a moist solid material with an absence of visible or free flowing water (Zweistra-Hoogschagen, 2006, Mitchell, *et al.*, 2006, Pandy *et al.*, 2001a, Raimbault, 1998). Generally this means a moisture level between 40-60%. Water is the critical element to all life and therefore essential to microbial growth. The limited water available in the process is often referred to and used as the definition of this fermentation, i.e., a fermentation in which free flowing water is absent. Water is therefore held tightly by the substrate, meaning that the water activity is lower than 0.99 a_w. Filamentous fungi can out-compete bacteria or yeast in this environment, as they

grow well at water activities between 0.93 a_w and 0.98 a_w (Corry, 1973), while bacteria and yeast prefer water activities above 0.99 a_w.

SSF presents different opportunities and challenges to conventional fermentation types. The impact of limited water is that the process of heat transfer becomes a major issue in SSF (Laukevics *et al.*, 1984). Evaporative cooling is an effective way of cooling, yet it further reduces the water activity to a point where the fungus may be challenged to grow (Trevelyan, 1974). A balance between these two factors must be found, so that the fermentation process does not overheat the koji and slow the fermentation rate, while at the same time the koji must not dry out, as that would also hamper microbial activity.

1.2.2 History of SSF

SSF has had a long history dating back over 4000 years. Its usage has been predominately in food production, although in more recent times applications of SSF for more technologically advanced products have been found.

1.2.2.1 Traditional products

SSF has been used for millennia in the production of various food products, particularly in Asia (Table 1.1). One example of this is soy sauce, a very traditional and recognisable taste in food from many Asian cultures, predominantly China and Japan. Soy sauce has at least 300 identified flavour components (Roberts, 2003).

Table 1.1 Production of foods using solid-state fermentation.

Product	*Microorganism*	*Materials*
Natto	*Bacillus natto*	*Soybean*
Tempeh	*Rhizopus oligosporus*	*Soybean*
Tape	*Amylomyces rouxii, Rhizobium chinensis*	*Rice, cassava, maize*
Ontjum	*Neurospora sitophila*	*Peanut meal*
Cheese	*Penicillium roqueforti*	*Wheat powder*
Bread dough	*Saccharomyces cerevisiae, Lactobacillus sanfranisco*	*Wheat powder*
Saké	*Aspergillus oryzae, A. kawachii*	*Rice, barley*
Miso	*Aspergillus oryzae*	*Soybean, rice*
Soy sauce	*Aspergillus sojae*	*Soybean, wheat*

Adapted from Mitchell and Lonsane (1992)

Soy sauce is produced by mixing soybeans, which are previously steamed and softened, and wheat, which is roasted and crushed, at a specific ratio and then fermenting it with a specific strain of fungus for three days. The recipe of the mixture of these two substrates and the specific organism, generally an Aspergillus, give each producer their own distinct soya taste and aroma. These Aspergillus strains produce extracellular enzymes including proteases and amylases which break down the starch and protein in the wheat and soybeans to create these flavours (Zweistra-Hoogschagen, 2006). This mass of material is called koji, and traditionally was fermented in large clay pots (Fig. 1.6). Today koji is fermented in large automated plants. After the first stage, salt dissolved in water, is added to the koji mixture and the resulting brew is called moromi. This addition slows the initial fermentation, and salt-tolerant lactic acid bacteria and yeast start to grow as the enzymes continue to break down the substrates. This mixture is further fermented in liquid fermentation vessels. Then, the liquid is pressed in order to release the final soy sauce product, which is refined and pasteurised.

Figure 1.6 Traditional soya sauce preparation.

Tempeh is another product that is produced through SSF. It originated on the island of Java in Indonesia several hundred years ago. Soybeans are fermented by a *Rhizopus oligosporus* strain. The fungus grows on de-hulled whole soybeans to create a cake which binds the beans together. The final product is the koji itself, which is used as a meat substitute. It is rich in nutrients and has a high protein content (Zweistra-Hoogschagen, 2006; Soccol, 1992).

Miso is a traditional Japanese food produced by SSF (Fig. 1.7). It is a rich and salty condiment and its flavour and aroma depend on both the ingredients used and the fermentation process. There are many variations of miso, but all are basically produced by fermenting rice, barley and/or soybeans.

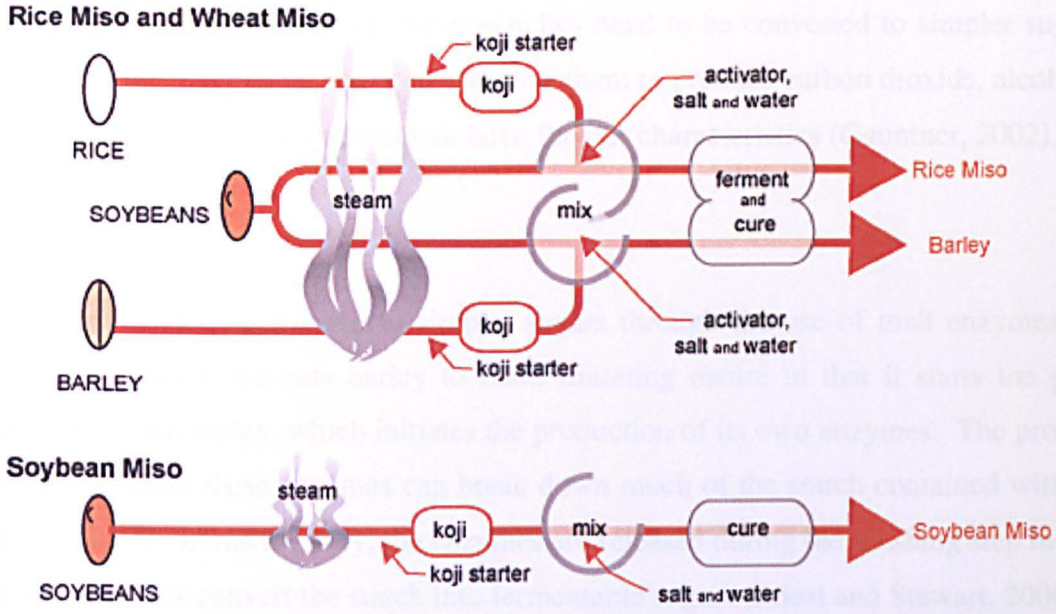


Figure 1.7 Miso fermentation process (adapted from Hidefusa, 2003).

Aspergillus oryzae is the most common fungus used for the production of the koji since it produces numerous enzymes capable of breaking down the proteins and carbohydrates of the starting raw materials. The miso ingredients are fermented and aged in wooden kegs for up to two years. There are over 1,400 miso-manufacturing plants in Japan and they produce over 560,000 tons of miso annually (Hidefusa, 2003).

In the west, production of blue cheese, through perforating fresh cheese and inoculating it with specific strains, is another form of SSF. Other mould cheeses are produced in a similar manner. Production of mushrooms and compost are also examples of SSF.

Saké is probably the highest value traditional SSF product. Saké is brewed from rice and its alcohol content is relatively high for a fermented product. It is produced initially to a strength of about 20% (v/v) alcohol, but this is usually diluted to about 16% (v/v) alcohol, which is higher than that found in most wines. However, saké is closer to beer than wine, in terms of its production. In the same way as beer is brewed, saké is brewed from grains and the processes have many similarities. Both barley (beer) and rice (saké)

contain complex starches and these starches need to be converted to simpler sugars in order for the yeast to be able to metabolise them to produce carbon dioxide, alcohol and other fermentation by-products that have flavour characteristics (Gauntner, 2002).

In beer, starch is converted to simpler sugars through the use of malt enzymes. The malting process converts barley to malt, imitating nature in that it starts the growth process of the barley, which initiates the production of its own enzymes. The process is stopped before these enzymes can break down much of the starch contained within the barley itself. In the brewery, the enzymes are released during the mashing step in which these enzymes convert the starch into fermentable sugars (Priest and Stewart, 2006).

In the case of saké, the husk-less rice cannot be malted as it has no enzymes present, so the enzymes are contributed by fungi known traditionally as koji-kin. The fungus which is used predominately is *A. oryzae*. This organism carries out the conversion process by producing extracellular enzymes, which break down the starch into sugars to be converted into alcohol.

The other principal difference between saké brewing and beer brewing, or any other brewing process, is that the starch-to-sugar conversion as well as the conversion of sugar to alcohol and carbon dioxide take place simultaneously, and in the same tank, and not sequentially, as in barley based beverages (Gauntner, 2002). This dual fermentation requires multiple organisms to work together and is unique in the world of fermentation.

The SSF process has started to become more modern and mainstream in terms of food production. Tempeh production has now been established on a small scale in the US (Hesseltine, 1987) because tempeh has been accepted as a meat substitute by vegetarians. Mushrooms are cultivated in western countries; and soy sauce production has become highly industrialised and is widely used across the world. Kikkoman Foods has built a state-of-the-art facility, completed in 1998, for soy sauce production in

Folsom, California and Kikkoman is now the largest user of SSF in the world.

1.2.2.2 SSF Biotechnology history

Thus for most of human history, SSF has been used for food production. Due to this tradition and familiarity of using SSF, it is not surprising that the most profitable non-food applications of SSF are in Asian and African countries, where SSF processes have been perfected over long periods.

In 1896, Dr. Jyokichi Takamine produced a digestive enzyme, Takadiastatse, by SSF employing *A. niger* on wheat bran (Takamine, 1914). This was the first microbial enzyme to be commercially produced in this manner, and many regard Takamine as the 'father' of the SSF process for biotechnological uses.

In western countries, traditional applications of SSF are scarce. SSF has been largely neglected since the 1940s and negligible research and development efforts have been made. The selection of submerged liquid instead of SSF in western countries was not based on economic comparisons of submerged liquid and SSF techniques; the choice was linked to slow growth of the microbial cultivation industries across the world (Ralph, 1976; Hesseltine, 1976).

While commercial use of SSF is not widespread in North America, industrial enzyme production by SSF has occurred for a number of years (Takamine, 1914; Underkofler *et al.*, 1958). After World War II, Underkofler *et al.* (1947) and Terui *et al.* (1957) used heaped bed cultures, a relatively crude but effective fermentation. This style was initially used around the 1900s for enzymes, and later for the production of penicillin, gibberellic acid, citric acid and limited production of antibiotics. Improvements were made once systems with forced aeration started to be used to produce enzymes and citric acid. However, today, SSF provides some important advantages and lends itself to specific applications in the realm of biotechnology and bioprocessing.

1.2.3 Submerged fermentation vs. solid-state fermentation (SSF enzyme energy benefit)

While microorganisms produce some of the most important products in daily life, from foodstuffs to antibiotics, most in the area of enzyme production have been grown in what is not always their most natural state. Most fungi are surface growers, however, the vast majority of the fermentation industry focused on fungal biomass or the compounds they produce using submerged fermentation (Zweistra-Hoogschagen, 2006). The reason that submerged liquid fermentation has been the preferred method for production of modern biotechnology products is clear. Performed in larger liquid fermenters, similar to those used for brewing, it generally produces products with higher yields, and the technology is easier to apply and is better understood (Mitchell *et al.*, 2006). Liquid fermentation has typically been favoured by engineers and technologists due to three principal factors: it is easier to control the temperature and pressure, to clean the vessels, and to modify parameters during the process. The level of solids in the liquid medium typically does not exceed 15% (El-Mansi and Bryce, 1999). Through the use of genetic engineering, yields have been significantly increased in submerged fermentations, above where they had typically been. Like many industries, the advantages of submerged fermentation have been further enhanced by the vast number of companies and researchers working on the process. Table 1.2 illustrates some of the differences between the two types of fermentation.

Table 1.2 Comparison of submerged and solid-state fermentation.

Parameter	*Submerged Liquid Fermentation*	*Solid State Fermentation*
Substrate type	*Soluble Substrates (sugars)*	*Polymer insoluble substrates, Starch, cellulose, pectins, lignin*
Aseptic conditions	*Heat sterilisation and aseptic Control (CIP)*	*Aseptic levels vary from clean rooms to open fermentation*
Water	*High volumes of water, significant effluent*	*Limited to moderate water consumption, clean effluent*
Metabolic heating	*Easy control of temperature*	*Low heat transfer capacity, cooling difficult*
Aeration	*Limited by dissolved oxygen capacity*	*Aeration flexible*
pH control	*Easy control of pH*	*Buffered substrates*
Mechanical agitation	*Good homogenisation, agitation*	*Static condition better*
Scale up	*Industrial systems in existence*	*Difficult and varied, unique designs*
Inoculation	*Easy inoculation, continuous process*	*Spore or mycelium inoculation, batch*
Contamination	*Single bacteria can spoil batch*	*Background bacterial growth tolerable, risk of fungal contamination*
Energy usage	*High energy consumption*	*Low energy consumption*
Investment and throughput	*High for both*	*Flexible for both*

There are many advantages and disadvantages that can be listed about SSF systems (Cannel and Moo-Young, 1980; Mudgett, 1986; Pandey *et al.*, 2001a; Mitchell *et al.*, 2006). Advantages are numerous, for example, potential substrates are often inexpensive and are by-products from other industries. Thus there is a good possibility of being able to add value to these materials. Also, the materials often have a good

level of nutrients already available for microbial growth. Substrates for SSF generally require little preparation for fermentation compared to submerged fermentation systems. Generally, only minor additions of nutrients are required. However, submerged fermentations often require a pre-treatment in order to extract the nutrients from materials. In SSF it is possible to control parameters such as initial pH and especially water, due to the lower level of water in the system, in order to aid the source organism to out-compete unwanted contaminants, particularly yeast and bacteria. Aeration is often easier since there are a lot of spaces within the system where air can enter. Downstream processing and pollution in the system is minimal and drying is made easier due to less water being present in the system.

Disadvantages include the following. There is a significant reduction in the number of organisms that can be used for fermentation due to the reduced moisture level involved in the SSF process. Metabolic heat removal can be very difficult on the large scale, but can also be a critical factor for the success of the fermentation. Combined with the first disadvantage this further reduces the number of candidate organisms that may be used. Basic scientific and engineering principals are currently poorly understood making the process very challenging. What knowledge there is has more of an academic focus than a practical one.

Due to the solid nature of the substrate, monitoring the fermentation is far more difficult than in a submerged fermentation and the number of parameters that can be monitored are fewer. Even when systems are developed to monitor parameters, control systems are often not available to be able to modify the specific parameter in the process. The process is often longer in terms of time to end of fermentation.

1.2.4 SSF production today

SSF has been modernised considerably from traditional processes. Much research is focused on bioreactor development for automation, as well as novel SSF products for niche markets, but the two key elements or improvements which receive the most attention are still concerned with improving the microorganism (with or without the use of genetic manipulation) and improving the design or technology for better control of heat removal.

1.2.4.1 SSF Products and Market

The market for SSF enzymes ranges from very specialised and niche products such as diagnostic enzymes to mainstream animal feed enzymes.

Companies working in the area of SSF include Shin Nihon Chemical Company and Amano Enzyme Inc. in Japan and Lyven in France. Together they would sell an estimated $200,000,000 in enzymes per year in established niche markets (Internal Alltech Report FC-RD 43, 2006). Examples of these would include pharmaceutical and diagnostic enzymes. Also, in some saké production, enzymes are added and all of these enzymes come from SSF enzyme production systems.

New interest in the area of SSF that can be found in the literature (Mitchell *et al.*, 2006; Pandy *et al.*, 2001a; Sweeten, 1991) focuses predominately on using waste products and upgrading their value. Beyond this many new opportunities for the technology exist, but have not yet been exploited.

1.2.4.2 Types of Bioreactors used for SSF

The varied types of bioreactors used around the world reflect the significant differences in the types of fermentations that fall into the area of SSF. Variation of substrate and organism are two of the options SSF technologists use to create desired end products. SSF type is the third critical factor to consider while designing a fermentation system.

There is much in the literature regarding different types of bioreactors for SSF (Durand, 2003; Krishna, 2005; Margaritis and King, 1971; Mitchell *et al.*, 2000; Pandey *et al.*, 2001a), but many of these reactors are not in use for industrial processes. Based on similarities in design, Mitchell *et al.* (2006) divided the various models into four groups based on how they are mixed or aerated. Figure 1.8 illustrates the four types of bioreactors.

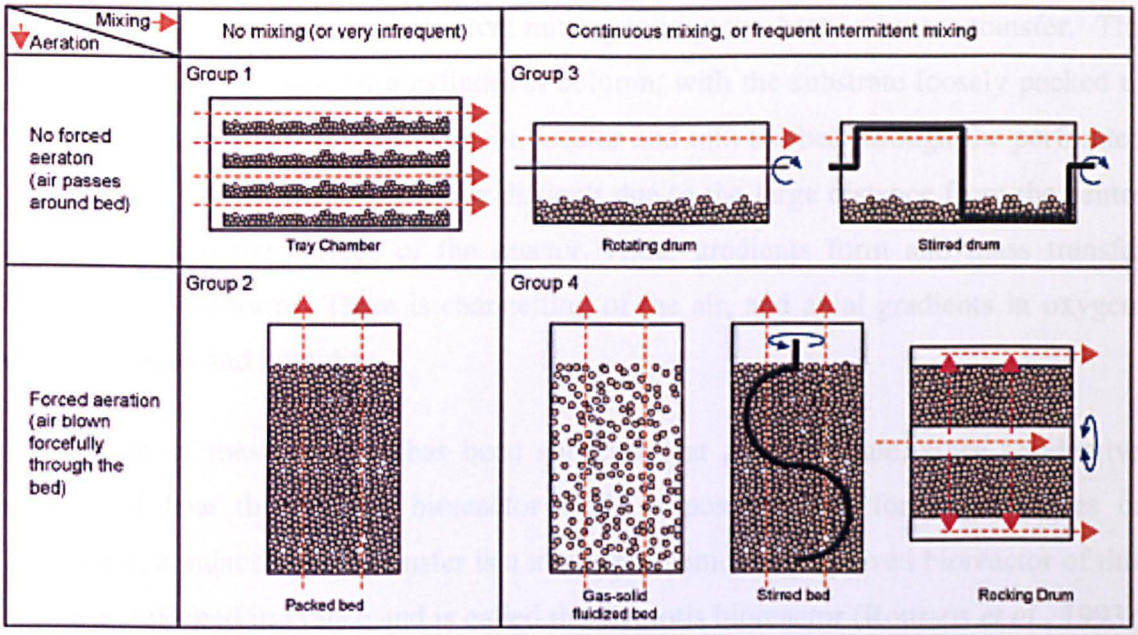


Figure 1.8 Grouping of bioreactors based on mixing and aerating (adapted from Mitchell *et al.*, 2006).

Group 1 Bioreactors (Tray and Chamber)

These are primarily the tray fermenters and are the simplest of fermenters used for SSF. The bed is usually static but could be mixed (at most once or twice a day). The substrate requires separate sterilisation. Air is circulated around the bed. The tray type of reactor maintains only a thin layer of solids (~5 cm). This reduces the distance from the solids to the gas phase, as well as the distance from the solids to the surface of the tray, for heat transfer. Trays are usually arranged one above the other with a suitable gap between them to allow air to circulate. Once the trays are inoculated, they are kept in a chamber where the humidity and temperature are controlled by circulating air or by spraying water throughout the fermentation. A system of this design is labour intensive, requires a large operating area, and to date has not been well automated. Scale- up can be achieved only by increasing the area of the individual tray or by using more trays. However, this process has been in use very successfully, for large scale SSF, by a number of companies for many years, especially in Japan.

Group 2 Bioreactors (Packed Bed)

These are primarily bioreactors in which the bed is static or mixed infrequently (at most

once per day). There is no mechanical mixing to improve heat and mass transfer. The reactor generally consists of a cylindrical column, with the substrate loosely packed to larger heights, and air is forced into the reactor and into the bed through the perforated plate on the bottom. Heat transfer is difficult due to the large distance from the centre of the solids to the surface of the reactor. Axial gradients form and mass transfer becomes non-uniform. There is channelling of the air, and axial gradients in oxygen, carbon dioxide and humidity.

The design of these reactors has been successful at a small scale, since conductive transfer of heat through the bioreactor walls is possible, but for larger pieces of equipment, conductive heat transfer is a major problem. An improved bioreactor of this type was designed in France and is called the Zymotis bioreactor (Roussos *et al.,* 1993). This equipment has internal heat transfer plates, but due to the presence of the plates, the reactor has lost the option for agitation for heat removal. The PlaFractor reactor design in India (Suryanarayan and Mazumdar, 2003), is a complicated tray type bioreactor consisting of horizontal trays stacked vertically in a vessel with a mechanical arm that permits mixing of the solids on each tray. Each tray has a heat exchange surface and ports for addition of air, nutrition and other flows. The Prophyta bioreactor, developed in Germany (Luth and Eiben, 2003), consists of a vessel in which horizontal trays are stacked vertically. Each tray has a cooling coil with vanes pointed upward into the solids. Air is supplied from the bottom and passed out at the top.

Group 3 Bioreactors (Rotating Drum Bioreactors and Stirred Drum Bioreactors)

These are bioreactors in which the bed is either continuously mixed, or is mixed intermittently, with a frequency ranging from minutes to hours. The substrate is in a rotating drum and air, rather than being blown forcefully through the bed, is blown through the headspace across the top of the substrate bed. The drum may also contain internal lifters to enhance the mixing action caused by rotation of the drum. With the rotating drum model, the whole drum, filled from 10 - 40% volume with substrate, rotates around its central axis to mix the bed. With the stirred drum reactor, the reactor is stationary and the paddles mounted on a shaft running along the central axis of the bioreactor rotate inside the drum. Uniform growth of the organism is possible in this type of fermenter, but mixing can lead to damage to the mycelia and thus affect the

process. Another difficulty found with this type of bioreactor is that the substrate particles can aggregate into a ball and this disrupts the fermentation process. However, the largest limitation of the bioreactor is its limited ability to remove the metabolic heat that forms during the fermentation (Pandey *et al.*, 2001a).

Group 4 Bioreactors (Gas Solid Fluidised Beds, Stirred Beds and Rocking Drum)

The gas – solid fluidised bed is a continuously stirred aerated bed. Gas is blown upward through a perforated base plate at a high enough velocity to enable good heat and mass transfer between the substrate and the gas phase (Nagel *et al.*, 2001). The continuous mixing makes this a less attractive option for filamentous fungi as many of these do not tolerate continuous mixing. For the stirred bed bioreactor, a mixer is included in the bottom to disrupt any substrate agglomerates. With the rocking drum bioreactor, substrate is held between two perforated drums lying horizontally and encased in a solid outer bioreactor shell. The two drums on the outside slowly rock back and forth. Intermittently-mixed forcefully aerated bioreactors are used for large scale industrial processes, in particular for the commercial production of soy sauce from koji (Mitchell *et al.*, 2006).

1.2.4.3 Microorganisms used for SSF

Several types of microorganism are used in SSF, although most utilise filamentous fungi, especially the genera Aspergillus and Rhizopus.

Aspergillus is one of the largest and most useful genera, with over 185 species. Several species of Aspergillus demonstrate oligotrophy, meaning that they are capable of growing in nutrient-depleted environments, or in environments where there is a complete lack of key nutrients. *A. niger* is a prime example of this phenomenon and can be found growing on damp walls and is a major component of mildew. It can even be found growing on whiskey warehouse and alcohol plant walls, feeding off the residual alcohol expunged from these facilities.

A. niger is a member of the black Aspergilli. In their natural habitat, *A. niger* strains secrete large amounts of a wide variety of enzymes, which release nutrients from the cellulosic materials on which the organism grows. For reasons not yet understood, *A. niger* is far more effective at secreting proteins than the yeast *Saccharomyces cerevisiae*. *A. niger* belongs to the family Trichocomaceae. The entire genome of this organism has now been sequenced (Pel *et al.*, 2007).

A. niger fermentation is used for metabolic production of many important industrial compounds including citric acid. Over 99% of global biological citric acid production is carried out using this organism. Many other carbohydrate modifying enzymes, such as gluconic acid and enzymes such as glucoamylase, α-galactosidase, lipases, glucanases, and proteases are produced using *A. niger*.

A. oryzae is an asexual, ascomycetous fungus used by humans for hundreds of years in the production of soy sauce, miso and saké. The organism has also been used to produce livestock probiotic feed supplements. It is well known for the significant amounts of amylases and proteases it secretes. Both *A. oryzae* and *A. flavus* belong to the section Flavi of the subgenus Circumdati of Aspergillus. However, *A. oryzae*, unlike *A. flavus*, does not produce aflatoxin, and has a long history of safe use in the food industry.

The commercialisation of by-products of the *A. oryzae* fermentation was established nearly a century ago by Dr. J. Takamine, who isolated and sold Takadiastase. The genome of this species of Aspergillus was initially kept secret, but was released by a consortium of Japanese biotechnology companies in 2005 (Masayuki *et al.*, 2005). The strain's eight chromosomes comprise 37 million base pairs and twelve thousand genes (Machida *et al.*, 2005). The genome of *A. oryzae* is one-third larger than that of *A. nidulans*, which has been used as the model organism for genetic studies. It is speculated that many of the extra genes present in *A. oryzae* may be involved in secondary metabolism. In addition, the genome of *A. oryzae* is highly enriched with

genes which are involved in biomass degradation (Kobayashi *et al.*, 2007). Despite the commercial importance of this fungus, very little has been studied on it since conventional genetic methods are difficult, due to the fact that it forms multinucleate conidia and lacks a sexual life cycle.

The genus Rhizopus contains several species and three common species are *R. oryzae* (*arrhizus*), *R. oligosporus*, and *R. stolonifer*. Rhizopus species generally grow well at 45° C and colonies of Rhizopus grow very rapidly and can fill a petri dish and mature in 4 days. The colony texture is typically cotton-candy like. Some Rhizopus species are agents of human zygomycosis and can cause serious (and often fatal) infections in humans and animals (Schipper, 1984). Under the microscope, nonseptate or sparsely septate broad hyphae (6-15 μm in diameter), sporangiophores, rhizoids (root-like hyphae), sporangia, and sporangiospores are visible.

Rhizopus species are often used in the production of fermented foods and alcoholic beverages in Indonesia, China and Japan. *R. oryzae* is used in the production of alcoholic beverages in parts of Asia and Africa. *R. oligosporus*, which is more properly known as *R. microsporus* var. *oligosporus*, is a fungus from the family Mucoraceae and is widely used in Asian food fermentations (e.g., the Indonesian staple tempeh). As discussed previously, in tempeh the spores produce a fluffy, white mycelium, which binds the beans together to create an edible "cake" of partly fermented soybeans. *R. oligosporus* also produces an antibiotic that inhibits Gram-positive bacteria (Masayuki *et al.*, 2005). This supports anecdotal evidence that those who eat tempeh regularly have fewer intestinal infections (Kobayashi *et al.*, 1992). *R. oligosporus* is a broad-based filamentous fungus found in soil, decaying vegetation and old foodstuffs and has a high occurrence in tropical and subtropical regions (Schipper, 1984; Soccol, 1992).

1.2.4.4 Parameters of SSF

Fungal hyphae develop a mycelial mat that grows over the surface of the bran particles. From this mat, aerial hyphae protrude both into the gas space, and into the liquid phase

in the pores of the bran. Most of the metabolic activities shown in the figure occur near the substrate surface and within the pores. The exposed regions of the mycelium, such as the aerial hyphae also metabolise the substrate and there can be a transport of substances from the penetrative to the aerial hyphae. Penetrative hyphae are those that have penetrated into the moist solid matrix. Aerial hyphae are those that are in direct contact with the air in the inter-particle spaces.

Hydrolytic enzymes are produced by the mycelium and these diffuse into the solid matrix and catalyse the degradation of macromolecules into smaller units, which can be taken up by the fungus for nutrition. Oxygen is consumed and carbon dioxide, water, heat and other compounds such as enzymes and secondary metabolites are produced during the fermentation. Gradients develop within the biofilm on the bran, and thus oxygen and carbon dioxide are forced to diffuse from the gas phase in the deeper region of the biofilm, towards the gas phase in the upper region. Heat development and removal is a major concern with solid-state fermentation. Heat is removed from the substrate not only via conduction but also by the evaporation of water. There is a delicate water balance. Water is taken up by the mycelium during growth, water is consumed during hydrolysis reactions, and there is water production by the fungus from respiration. The pH in the bran is also affected by the release of carbon acids and the exchange with ammonia. Enzymes of interest such as phytase and protease are released into the solid matrix and liquid filled spaces by the fungus.

Figure 1.9 is a schematic that illustrates the many micro scale processes that take place during solid-state fermentation.

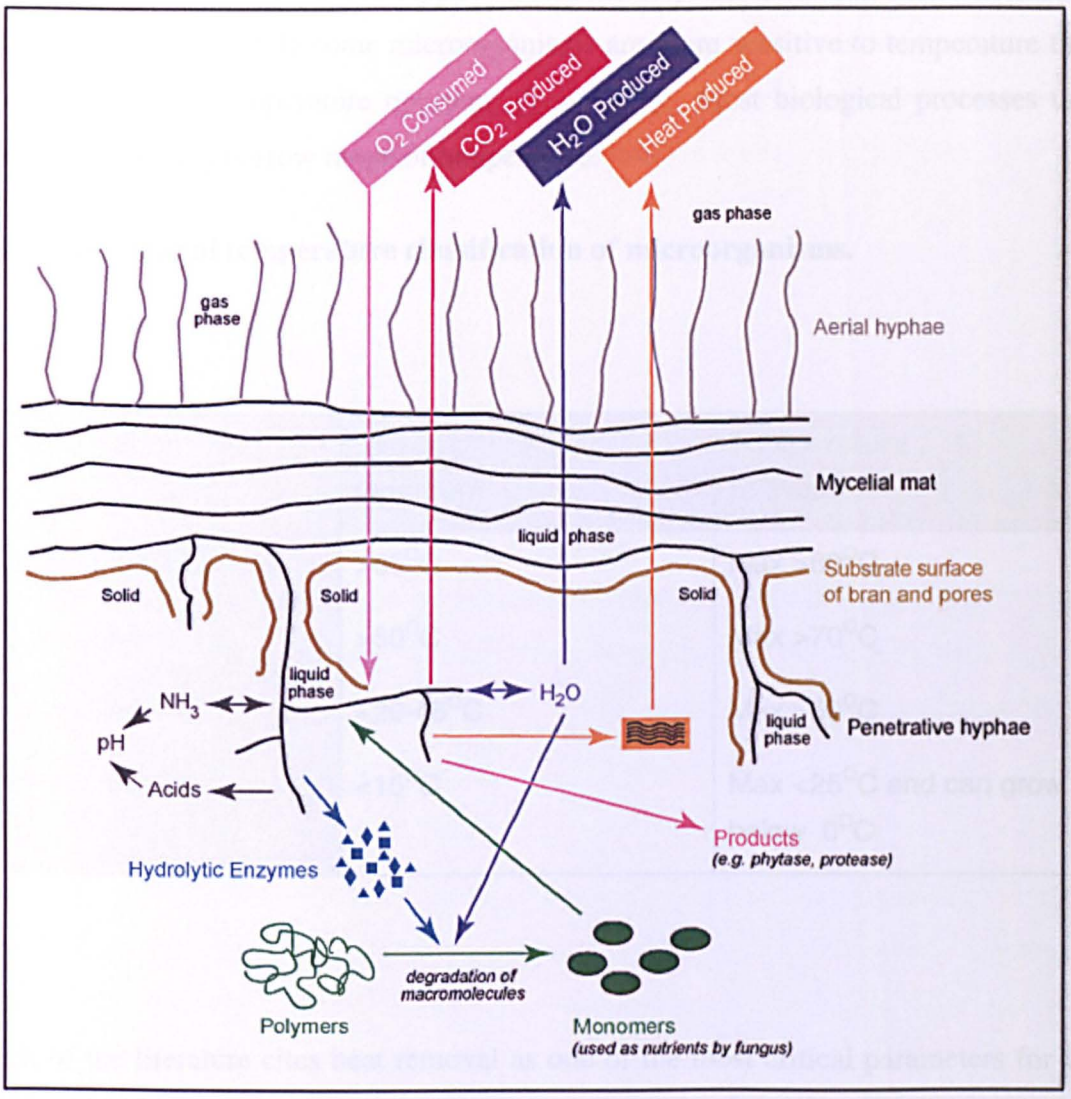


Figure 1.9 Microscale processes that occur during solid-state fermentation (adapted from Holker and Lenz, 2005).

There is also a macro scale view of the process and from this perspective one has to consider the bioreactor wall (with the process discussed in this project the metal tray and lid holding the koji), the headspace above the tray which is full of gas, and the substrate bed (bran particles) and air in the inter-particle spaces.

The bioreactor wall acts as a partial barrier and energy crosses the barrier by conduction and can be stored in the wall and thus increase in temperature. To control the conditions inside the substrate bed we are limited to manipulating variables outside the

reactor such as temperature. Temperature control is paramount to a successful solid-state fermentation. While some microorganisms are more sensitive to temperature than others, each has a temperature optimum (Table 1.3). Most biological processes take place in a relatively narrow range of temperature.

Table 1.3 General temperature classification of microorganisms.

Classification	Temperature Optimum	Temperature Extreme
Extreme thermophiles	>65^{o}C	Max >80^{o}C
Thermophiles	>50^{o}C	Max >70^{o}C
Mesophiles	~20-45^{o}C	Max >50^{o}C
Psychrophiles	<15^{o}C	Max <25^{o}C and can grow below 0^{o}C

Much of the literature cites heat removal as one of the most critical parameters for the fermentation. Since there is no free flowing water in the solid-state fermentation and relatively low thermal conductivity in the solid fermentation material, one of the major issues with scale up involves temperature control and heat removal (Zweistra-Hoogschagen, 2006). High temperature gradients can significantly impact microbial growth and enzymatic activity. In addition, the optimum temperature for growth can be different from that for product formation.

One of the most difficult problems in dealing with a solid-state fermentation is that throughout the fermentation the fungus is consuming nutrients and oxygen, and releasing waste, which alters the local pH, as well as releasing waste metabolic heat. Thus, although we are trying to control the conditions within the koji, the substrate is constantly changing away from the optimal values with which the fermentation was started. Thus, the specific transport phenomena within the bed will determine the

effectiveness of attempts to control the interior of the reactor. The cell physiology is difficult to study and the interactions between the environment and the process are difficult to control. Compared to our understanding of the liquid fermentation process, we understand very little about the solid-state process (Mitchell *et al.*, 2006).

1.3 Outline of Thesis

The general aim of this research project was to acquire more fundamental knowledge about the SSF process, about the issues of scale up from a 200 ml koji flask to a 10,000kg production scale and how to manipulate parameters such as temperature, moisture, pH and nutrition on a plant scale to enhance the profitability and productivity of the process. This knowledge should lead to overall recommendations for design of experiments that will more accurately define optimum plant fermentation parameters.

In Chapter 1 "Introduction" an overview of the use of enzymes in the feed industry is discussed and emphasis is placed on the enzyme phytase and its history and importance. Basic definitions of SSF and a history of the koji process are reviewed. The emerging new market for SSF products is addressed and why there is so much new interest in SSF at this time. Differences between liquid fermentation and SSF are explored and the benefits of SSF are discussed. Innovations in terms of new fermentation systems are covered, as well as which organisms are most popular for SSF fermentations and why they were selected. The microscale processes that occur during SSF are explored and the complexity of the system is discussed.

In Chapter 2 "Methods" the first section is a description of the SSF commercial process and it takes the reader through the steps from selecting the fungal strain, to facilitating its growth on plates, to scale up to liquid culture, and finally to large plant scale SSF. The origin of the strains and the testing to confirm their identity, including some electron microscopy is given. The next section of "Methods" focuses on the input to the fermentation: water, wheat bran, and additional nutrients.

Contamination is a major problem with SSF since it takes place over a 3-4 day period in an environment that is moist and warm. The sanitation procedures to overcome microbial issues are discussed in detail, including such parameters as operator access to the fermentation chambers. Standardisation of sampling techniques and standard analyses are documented in the "Methods" section.

Chapter 3 is "Results" and the first section focuses on standardisation and exploration of variables in the experimental and sample techniques and this is followed by the manipulation of environmental factors to attempt to improve the production process and final enzyme yield. Defining what is expected in terms of improvement in a process and being able to show statistically that the addition of substrates x, y or z, alone or in various combinations along with changes in temperature, moisture and pH, have a positive or negative effect requires the use of a number of statistical techniques. The use of a nested design and the blocking technique was useful in establishing what the "noise" level was in the process. The use of quadratic equations to look at multiple variables at one time allowed the progressive prediction of what parameters could be optimised in the plant.

Most of what is in the literature on SSF has been carried out on a small scale in research laboratories in universities and research institutes. Little has been published by companies detailing their actual processes, thus there is a lack of literature available in terms of actual industrial processes.

This thesis has focused on the industrial process in place in Serdán Mexico and on how to optimise a process that involves koji trays in fermentation chambers.

Although there is much in the older literature on different kinds of bioreactors, few if

any of these are in use today for the large scale production of ingredients that are not high value pharmaceutical type products. At this time, tray fermentation is still seen as the only option for the type of animal feed products that Alltech produces in Serdán. Other systems are not yet proven, are not sterile enough for the sensitive organisms used, or are too expensive.

The first problem that needed to be tackled was to gain a better understanding of the mass and energy balance in the system in the plant. Previous research had been carried out in conjunction with a local university defining the plant parameters. An overview of this is covered in the section on mass and energy balance and there is a discussion of the problems in water and air supply and heat removal from large scale incubation chambers (section 3.1).

The past research experiments looking at additions to the substrate or modifications of fermentation parameters conducted in the plant could never be repeated on a consistent basis. They lacked good statistical analysis and experimental set up. Thus changes that could optimise the process were too often implemented based on a 'one off' experiment that, if they had been repeated, would in fact have given the opposite result. This means that no conclusions should have been drawn, since a trustworthy system was not in place to validate experimental work in terms of repeatability, accuracy, and precision. Consequently in the thesis work, significant energy and time were expended on the process of sampling and technique standardisation. This included looking at such factors as sampling reproducibility, enzyme assay reproducibility and drying of the substrate before analysis (Section 3.3).

An examination was also carried out to see if the other enzyme products co-produced by the fungus were directly correlated to the production of phytase and protease. Four different fungi were used in the study, but the majority of the work focused on the production of phytase by the strain *Aspergillus niger* and the production of protease by the strain of *Aspergillus oryzae,* since these enzymes were the two key industrial

products produced by the plant on a daily basis (Section 3.3.5).

When investigating SSF, it is important to remember that the first part of the process is in fact a liquid process. There is also scope for improvement in this area, but this was not a focus in the thesis, as other laboratories have worked on this segment of the process, and consistency of inoculation appears to be more important than the actual dilution factor employed, so standardising to one technique was key, as it removed another variable from the process (Section 3.4).

The examination of the substrate was also a key learning process. Effect of wheat bran source on enzyme activity, effect of the particle size and bran depth, the environment (location in the chamber where the tray was held) and even effect of parameters such as light on the culture were examined (Section 3.5).

Once there was confidence that the assay and the substrate and the environmental factors could be standardised, or at least controlled to some degree and variation measured, and factors such as the ability to remove heat well understood, experiments looking at single variable optimisation were carried out.

There are many reports in the literature on the effects of supplementation of solid media substrates, but the literature is conflicting, which suggested that it was probably strain and specific media dependent. The effect of nitrogen and carbon addition are discussed and experiments were carried out to confirm past plant/other laboratory experience that rather than enhancing enzyme production, mostly the additions were either detrimental or had no effect, but would add additional cost and labour to the process (Section 3.6.1.1 and 3.6.1.2).

The exception was the supplementation work employing a yeast extract product

produced by Alltech. This was a cost effective source of nutrients that could be used in the plant with no negative customer perceptions and could enhance the feed product (Section 3.6.1.3).

In addition to supplementation of the koji, one could manipulate the process by altering the pH of the koji (but only initial pH, as it is too difficult to modify pH in solid media once the substrate is in the tray and virtually inaccessible) or by adjusting the fermentation temperature. Moisture control is also of interest but more difficult to do on a large scale than on the koji flask scale (Section 3.6.2.1). The effect of manipulating the temperature was investigated as this is an option for controlling the fermentation, although the main issue is being able to remove the heat rapidly enough during the active stage of fermentation. Again, this is a difficult parameter to examine in the koji flask, as the environmental parameters and heat transfer issues are so very different from what is found on a tray with a lid (3.6.2.2). It is also difficult parameter to study in a plant incubation chamber, as there is a maximum cooling capacity built into the current system, although these experiments led to changes in that cooling capacity.

It was also of importance to understand the growth behaviour of both the phytase and protease culture. Time course experiments were conducted, both in flasks and in the chambers. Cultures were allowed to ferment for longer periods, but with phytase although levels can continue to increase, issues then develop with contamination. With protease, the activity decreases with longer incubation times (Section 3.6.2.3 and 3.6.2.4).

The last section of the thesis focuses on using a better procedure for optimising experimentation both in koji flasks and in plant large scale trays. Single variable experiments are of value to give direction, but in a biological system there are many other interactions, and these cannot be ignored. Thus multiple variable and quadratic optimisation techniques were employed and again things that were controllable, such as

pH and moisture and nutrients were examined, but this time looking at how they interact. One of the main problems encountered in the thesis work was the variance that was constantly seen and the optimisation experiments with the advanced statistics revealed that indeed some of these interactions would not be detected in single variable experiments and that using the process of optimisation for the system would allow better decisions to be made based on sound statistics (Section 3.7).

Chapter 4 is an overall discussion and the aim of this chapter is to show what the challenges are in SSF and what experimental design will give in terms of direction and what the opportunities are for this technology. Although optimising a large scale process in a production plant is always limited by financial constraints, as well as equipment constraints, massive improvements in sterility, productivity and knowledge of the process have occurred over the past four years and techniques have been developed that will allow further improvement in the process of SSF.

Chapter 2 Materials and Methods

2.1 SSF – Commercial Process Description

The SSF system used in this study is located in the Alltech plant in Cuidad Serdán in the state of Puebla in Mexico. The town of Serdán is located between Mexico City and the port of Veracruz on the Gulf of Mexico. The plant has been in operation for seven years and has gone through several modifications and upgrades, focused on optimisation.

The Solid- State Process used in the plant is divided in four main stages: seed propagation, liquid fermentation, solid fermentation and lastly downstream processing.

The propagation stage and enzyme assays were performed in a 225 m^2 laboratory. The laboratory includes two clean rooms with HEPA filtration and laminar flow hoods for the transfer and inoculation work and laboratory equipment as detailed later.

The liquid fermentation area is 200 m^2 and the equipment consists of six 40 L stainless steel fermenters (third seed or S3), three 1000 L stainless steel fermenters (fourth seed or S4) and a 4,000 L stainless steel dilution tank, a mobile cleaning-in-place (CIP) system, sanitary pipes and valves, and numerous instruments to control and register all process variables.

The solid fermentation area is 10,000 m^2 consisting of six fermentation chambers and a downstream processing facility which includes two fluid bed dryers, and grinding and packaging equipment. A flow diagram of the process, as it is conducted in the plant in Serdán Mexico, is illustrated in Figure 2.1.

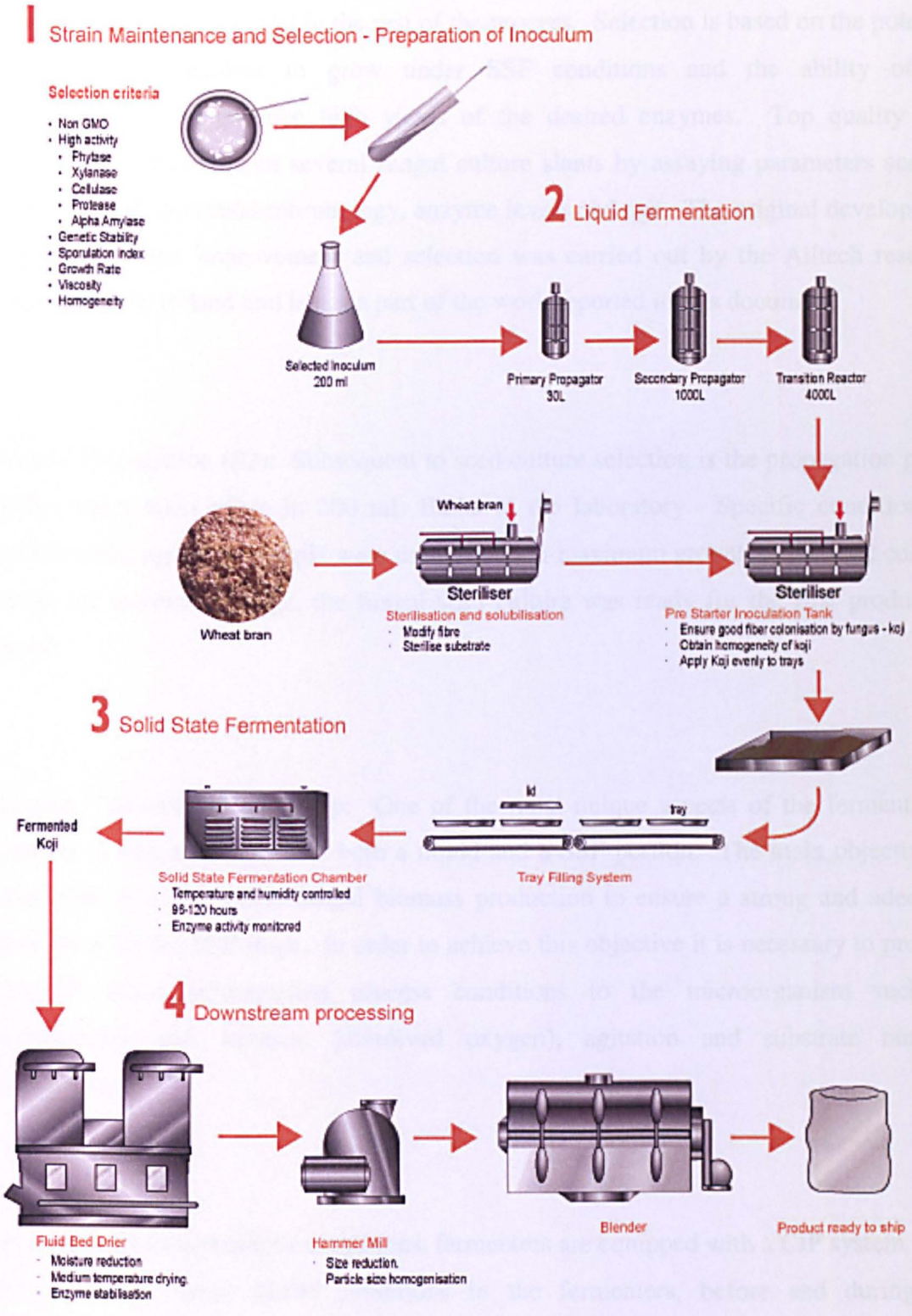


Figure 2.1 Flow chart of the SSF process showing an overview of the detailed stages of the process.

Seed Culture Propagation (S1): The first stage of the procedure is key for the success of the entire SSF process. The objective is to select and propagate the best microorganisms to be used in the rest of the process. Selection is based on the potential of the microorganism to grow under SSF conditions and the ability of the microorganism to produce high yields of the desired enzymes. Top quality seed cultures are chosen from several fungal culture slants by assaying parameters such as growth yield, microbial morphology, enzyme levels and age. The original development work on culture improvement and selection was carried out by the Alltech research department in Ireland and is not a part of the work reported in this document.

Liquid Propagation (S2): Subsequent to seed culture selection is the propagation phase (S2), which takes place in 200 mL flasks in the laboratory. Specific conditions of temperature, agitation and pH were used to obtain maximum growth of the seed culture. After the incubation stage, the fungal seed culture was ready for the first production stage.

Liquid Fermentation Scale Up: One of the most unique aspects of the fermentation process is that it incorporates both a liquid and a SSF portion. The main objective of this stage is to maximise fungal biomass production to ensure a strong and adequate inoculum for the SSF stage. In order to achieve this objective it is necessary to provide optimal liquid fermentation process conditions to the microorganism such as temperature, pH, aeration (dissolved oxygen), agitation and substrate nutrient composition.

In order to maintain aseptic conditions, fermenters are equipped with a CIP system. It is important to ensure sterile conditions in the fermenters, before and during the fermentation cycle, as this can be one of the main risks in this area. It is essential to eliminate any risk of contamination by other microorganisms that could compete with the production culture and reduce final enzyme yield.

The fermenters in the S3 stage have a total capacity of 40 L and a working volume of 30 L. Figure 2.2 illustrates the battery of 30 L fermenters employed for the scale up procedure.

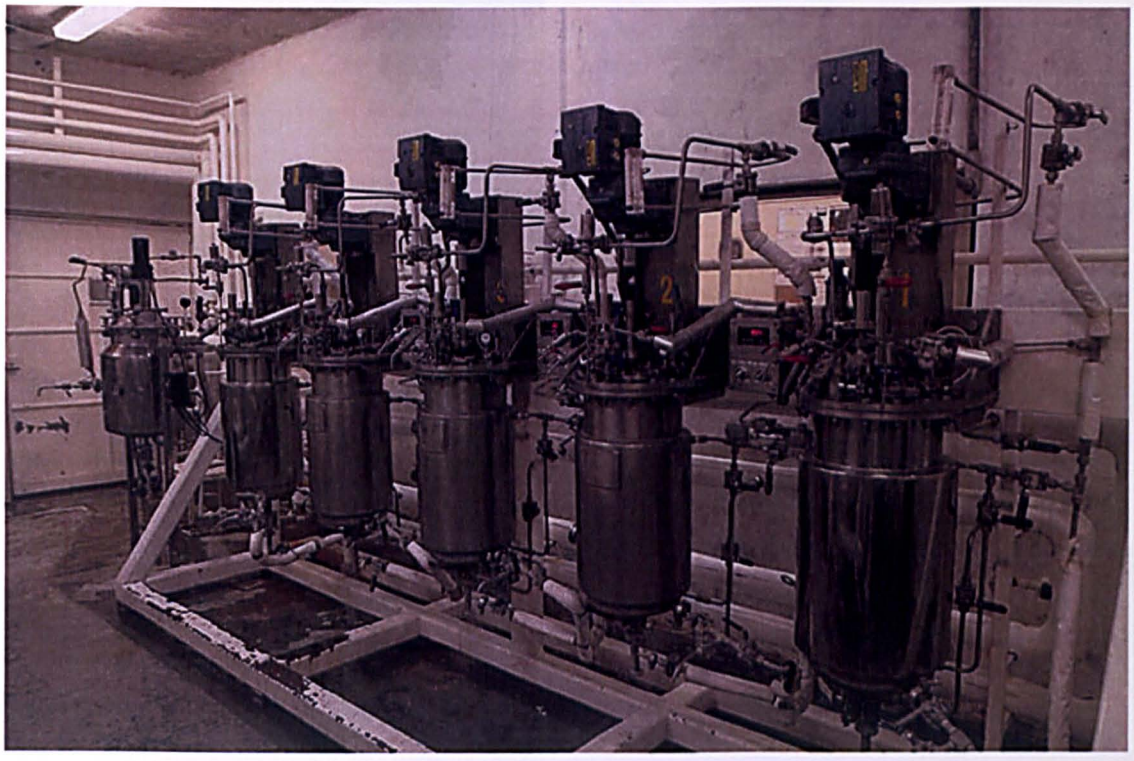


Figure 2.2 Stage 3 propagation – battery of 30 L fermenters employed for liquid scale up.

These fermenters were used for the propagation of fungal mycelium in a liquid medium for 36 h. The fermentation culture (S3) was transferred to a 1,000 L fermenter (this stage is termed S4) for 16 h to increase fungal biomass and to generate the volume required for the solid medium fermentation incubation stage (Fig. 2.3).

After S4 fermentation, the inoculum was transferred to a 4,000 L tank, and diluted with sterile water to achieve increased volume and an optimal cell concentration for wheat bran inoculation (essentially a 1:4 dilution).

Figure 2.3 Fermenter for S4 scale up phase (1000 L)

Solid- State Fermentation: This stage of the process is the main difference between SSF enzyme production when compared with the more common process of submerged fermentation. The objective in this phase is to produce the required enzymes on a solid substrate medium with optimal substrate conversion and the highest possible production of required enzymes.

After the liquid fermentation stage the diluted inoculum was mixed with sterile wheat bran in a patented steriliser mixer system. Once the wheat bran was sterilised and mixed with the inoculum, it was unloaded using a belt conveyor that fed into a hopper. Another belt conveyor below the hopper emptied the product onto sterile aluminium trays, which were then covered with sterile lids (Fig. 2.4).

Figure 2.4 Product being filled onto trays via a conveyer belt out of the koji hopper.

Each tray received ~2.8kg of wet product with even layer at a bed depth of ~3.5 cm. The belt filled the trays at a rate of 10 trays per minute. After the trays were placed onto the trolleys, they were manually moved into the culture chambers. The tray dimensions (Fig. 2.5) and tray loading (Fig. 2.6) are illustrated below.

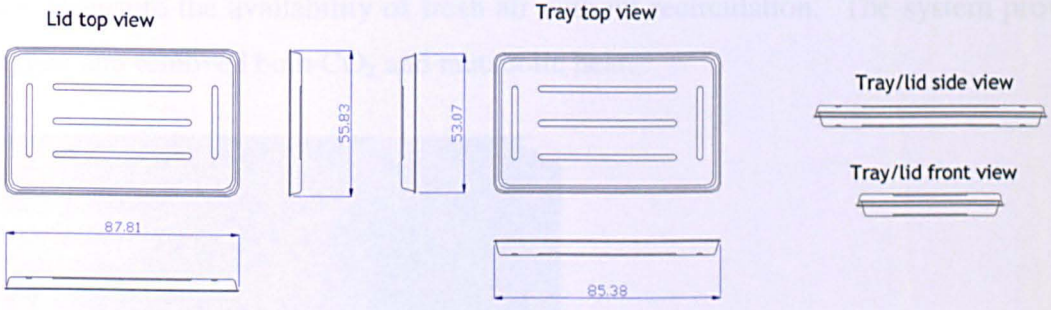


Figure 2.5 Schematic of koji trays and lids (sizes and views).

Figure 2.6 Photo of koji trays loaded with wheat bran.

Solid substrate fermentation in the culture chambers took four days and the koji temperature was controlled at 30-32°C using spray water nozzles located in the ceiling. The heat and CO_2 generated during the fermentation were removed with sterile air provided by the air conditioning system (Fig. 2.7). The air conditioning system was used to ensure the availability of fresh air without recirculation. The system provided oxygen and removed both CO_2 and metabolic heat.

Figure 2.7 Air ducts and filter unit on roof of fermentation chamber.

The equipment that makes up the air handling system consisted of one blower and one extractor for the six culture chambers. The incoming air was sterilised through a bank of filters (pre-filters, bag filters and HEPA filters). Figure 2.8 illustrates the water and

air flows in the process.

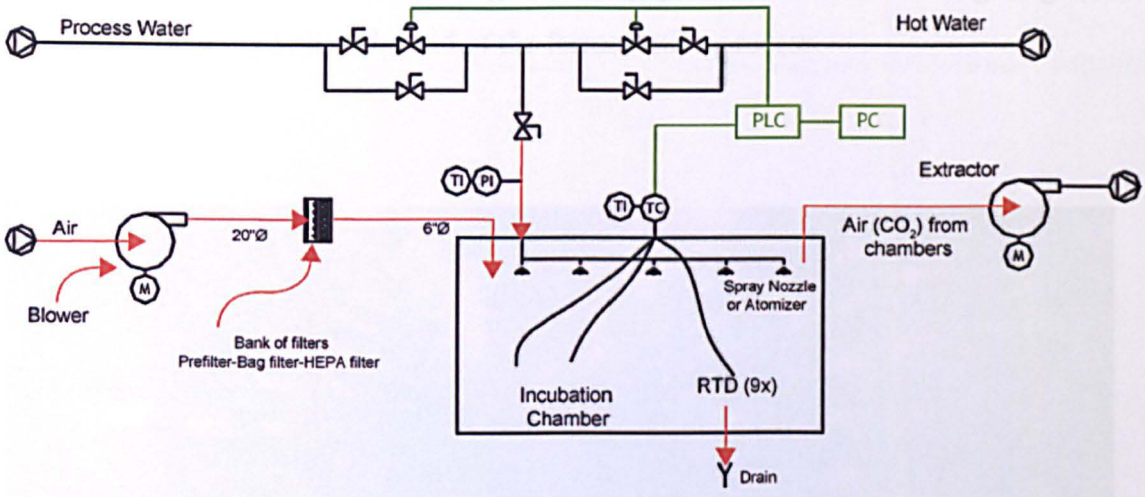


Figure 2.8 Water and air distribution system in the culture chamber.

Air was distributed via isolated ducts, which have three inlets per chamber, without diffusers. The dimensions of the ducts vary from 48 cm close to the filters, to 15 cm for air injection inside the chamber. Each chamber had an air flow of approximately two to three changes per hour (cph), but this number varied depending on other process parameters. The O_2 and CO_2 levels in the culture chambers were monitored intermittently.

The temperature of the koji was regulated using hot or cold water. Each chamber had nine stainless steel pipes with 45 water nozzles and a total water flow capacity of 5,110 L/h. At the beginning of each fermentation cycle, hot water was sprayed in order to achieve the target temperature of 32°C as quickly as possible.

After the fermentation was complete, trolleys were removed from the culture chambers (Fig. 2.9) and moved to the downstream processing stage. Up to 30% of the wheat bran is typically lost due to metabolism during fermentation.

The storage capacity for each chamber is 55 trolleys i.e., 2,695 trays of product with lids. This is the equivalent of 7,540kg of wet koji product (48% moisture) giving 2,750-2,950kg of final dry koji at the end of the fermentation process.

Figure 2.9 Typical incubation chamber loaded with trolleys.

The majority of the heat generated during the fermentation was removed by spraying cold water. The pneumatic valves (hot/cold) were controlled by a PLC (Allen Bradley, Milwaukee, Wisconsin) linked with nine resistance thermal devices (Pt 100 RTD) per chamber and these read the temperature directly from the product trays. The control set point was adjusted to ~32°C and the PLC system controlled the water system to keep the temperature in the trays at the set point. Figure 2.10 shows the typical temperature profile of a 4 day fermentation.

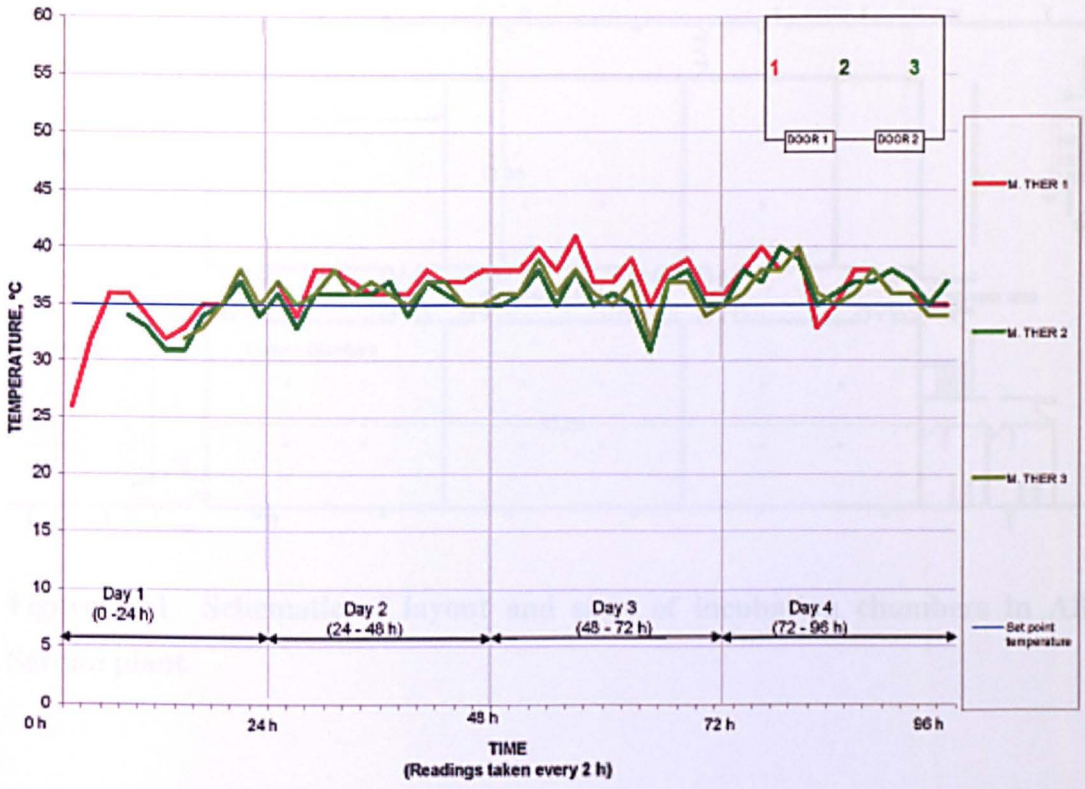


Figure 2.10 A typical koji temperature profile over a 96 h fermentation.

Aluminium trays and lids were used to support and cover the koji during the fermentations. Trolleys were fabricated from aluminium and stainless steel (SS 316). Each trolley had a loading capacity of 48-50 trays. The spacing between the trolleys was variable and depended on the number of batches in the chamber.

Alltech Serdán has six culture chambers laid out in two parallel lines with a central inlet/outlet hallway that connected to the tray filling area and to the downstream area.

Chamber dimensions are as follows: width and length 11.0 m x 13.9 m, height 3.5 m and volume 539.0 m^3. Each chamber has two sliding doors (inlet/outlet) and waterproof lighting (Fig. 2.11) and trolley loading can be seen in Figure 2.12.

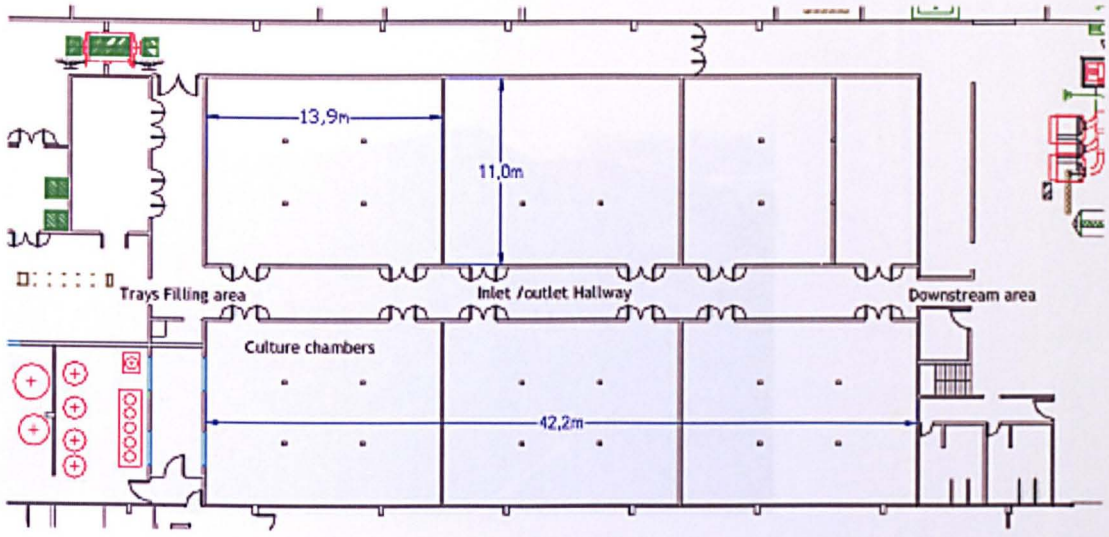


Figure 2.11 Schematic of layout and sizes of incubation chambers in Alltech Serdán plant.

Figure 2.12 Trolley loading from inlet/outlet hallway into an incubation chamber with an operator setting the temperature program.

Building construction was based on concrete blocks mounted on a metallic frame. The finish was smooth polished concrete. Figure 2.13 is a view of the back of the building that houses the fermentation chambers.

Figure 2.13 Photo of building housing the fermentation chambers.

After four to five days of solid substrate fermentation in the culture chambers, the koji was processed in the downstream area using a fluidised bed dryer (FBD) (Horizon Systems Inc., Lawrance, Kansas). The koji was conveyed on a belt conveyor from the trays to the FBD, where the moisture level was reduced from ~50% to ~10%. Product was dried using a hot air flow heated by steam and controlled at 42°C as the maximum temperature. The air flow comes in from the bottom of the unit lifting the material and creating a fluid movement in the material. Bag-houses are used at the outlet of the air to avoid any losses of product.

After drying, the product was milled and blended to produce a product with homogeneous enzyme activity and particle size. Production product for sale was then sampled for approval by Quality Control, packaged and labelled. The sampling is carried out using a sample rod. Enzymatic activity is used as the main quality control parameter. Product is packaged in 750kg bags and shipped to the plant in Nicholasville, Kentucky for final quality control and packaging in 25kg bags for shipment to the customer.

2.2.1 *Strain background and preservation*

Four production fungal strains were employed in this study (Table 2.1). They were as follows: a proprietary strain of *A. niger* for phytase production; a proprietary strain of *A. oryzae* for protease production; a proprietary strain of *R. oliogosporus* for commercial protease and glucoamylase production; and a proprietary strain *R. oryzae* for commercial glucoamylase production. The identity of the fungal strains was confirmed by the University of Nuevo Leon, Mexico and Microcheck Inc., USA. These proprietary strains were originally sourced from Japanese and Russian industrial processes and all four underwent an in-house strain improvement program carried out by the Alltech laboratory in Dunboyne, Co. Meath, Ireland.

Table 2.1 List of strains.

Genus and species	Internal code	Enzyme of key interest	Original source of strain	Strain improvement carried out
Aspergillus niger	PHY–ML	Phytase	Japan	Yes
Aspergillus oryzae	PRO–ML	Protease	Russia	Yes
Rhizopus oligosporus	Gluc1–ML	Protease and Glucoamylase	Japan	Yes
Rhizopus oryzae	Gluc2–ML	Glucoamylase	Japan	Yes

The strains are maintained by storage on Potato Dextrose Agar (PDA) (Difco Laboratories, Detroit, Michigan) slopes at 4°C. Working slopes were further sub-cultured every two weeks. Master cultures of the strains are stored as freeze-dried vials in the Alltech laboratory in Dunboyne, Co. Meath, Ireland.

2.2.2 *Electron microscopy*

Production strains *A. niger* and *R. oryzae* were sent for electron microscopy at the University of Guelph, Ontario Canada. This was conducted as an additional method of identifying strains as well as giving a visual example of the growth of the organism on the wheat bran substrate. An inoculum from master culture slopes was precultured in liquid S2 medium for two days and then inoculated into a 200 mL koji flask using standard plant procedures and allowed to grow for three days. A 25 g aliquot of the culture on bran from the koji flask was placed into a test tube and sent for electron microscopy analysis. Upon receipt, the samples were preserved overnight in 2% glutaraldehyde (in water). The next day the samples were rinsed in Sorenson's phosphate buffer (pH 6.8) and placed into a solution of 1% OsO_4 (in water) for 1 h. Samples were rinsed in phosphate buffer and then dehydrated in a graded series of ethanol concentrations. The samples were critical point dried (Ladd brand dryer), mounted on stubs with carbon tape, coated with gold/palladium in a Polaron sputter coater and examined using a Hitachi S-4500 SEM. Digital images were captured using Quartz Xone.

The morphologies of Aspergillus and Rhizopus strains growing on wheat bran were used to confirm the identification of the strains using electron microscopy. In Figure 2.14 and Figure 2.15 the conidia (asexual spores) can be seen on the conidiophores (fruiting structures/sporangia) of the Aspergillus strain. The asexual sporangiospores of Aspergillus are very distinctive.

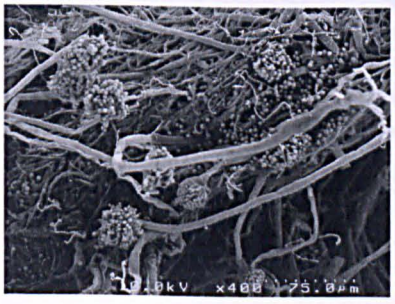


Figure 2.14 Structure of *A. niger* on wheat bran at 400X magnification.

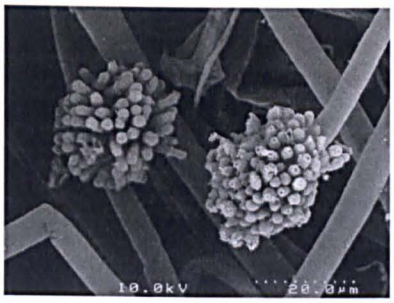


Figure 2.15 Structure of *A. niger* on wheat bran at 1500 x magnification.

With the Rhizopus strain a very different morphology was observed (Fig. 2.16 and Fig. 2.17). Rhizopus produces asexual and sexual spores. The sporangia are supported by a large apophysate columella and the sporangiophores arise among distinctive rhizoids.

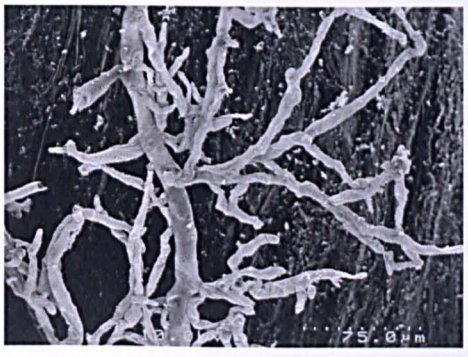


Figure 2.16 Structure of *R. oryzae* on wheat bran at 400 X magnification.

Figure 2.17 Structure of *R. oryzae* on wheat bran at 1500 X magnification.

2.3 Water

Water plays a very important role in SSF. It must be present in the right amounts and in the right places. As one of the principal vectors of contamination, it must also be sterile.

2.3.1 Source

The water at the plant in Serdán is drawn from a well and treated in-house. Analysis of the water can be seen in Table 2.2.

Table 2.2 Alltech Serdán well water analysis (expressed as mg/L where applicable)

Parameter		Parameter	
pH	7.5	Nitrates	1.12
Total Alkalinity	190	Nitrites	0.013
Total Hardness	146.60	Ammonia Nitrogen	0.19
Hardness (Calcium)	132	Aluminium	0.20
Hardness (Magnesium)	24	Arsenic	0.002
Chlorine (as Cl)	7.07	Barium	0.70
Sulfate (as SO_4)	6.54	Cadmium	0.003
Silica (as SiO_2)	56.2	Cyanides	0.07
Total Phosphates (as	1.1	Chlorides (Free)	0.80
Orthophosphate (as PO_4)	0.39	Copper	0.048
Polyphosphate (as PO_4)	0.71	Chromium	.045
Iron (as Fe)	2.7	Sodium	50.00
Conductivity (microohms)	314	Zinc	3.00
Total Suspended Solids	100	Manganese	0.15
Colour	Colourless	Fluorides	1.50
Odour	No odour	Mercury	0.001
Appearance/Turbidity	0.5	Lead	0.009
Phenols	0.018	Phenolic Compounds	0.30
Fluorine	0.82	Coliforms	Negative

2.3.2 Treatment

Water used for the SSF process is treated in a number of ways depending on the ultimate use in the plant. Figure 2.18 illustrates the water system in the Serdán plant. For the chambers, the water must be cooled and sterilised before use. This is achieved through the use of cooling towers. Figure 2.18 illustrates the water process flow from the well to the culture chambers.

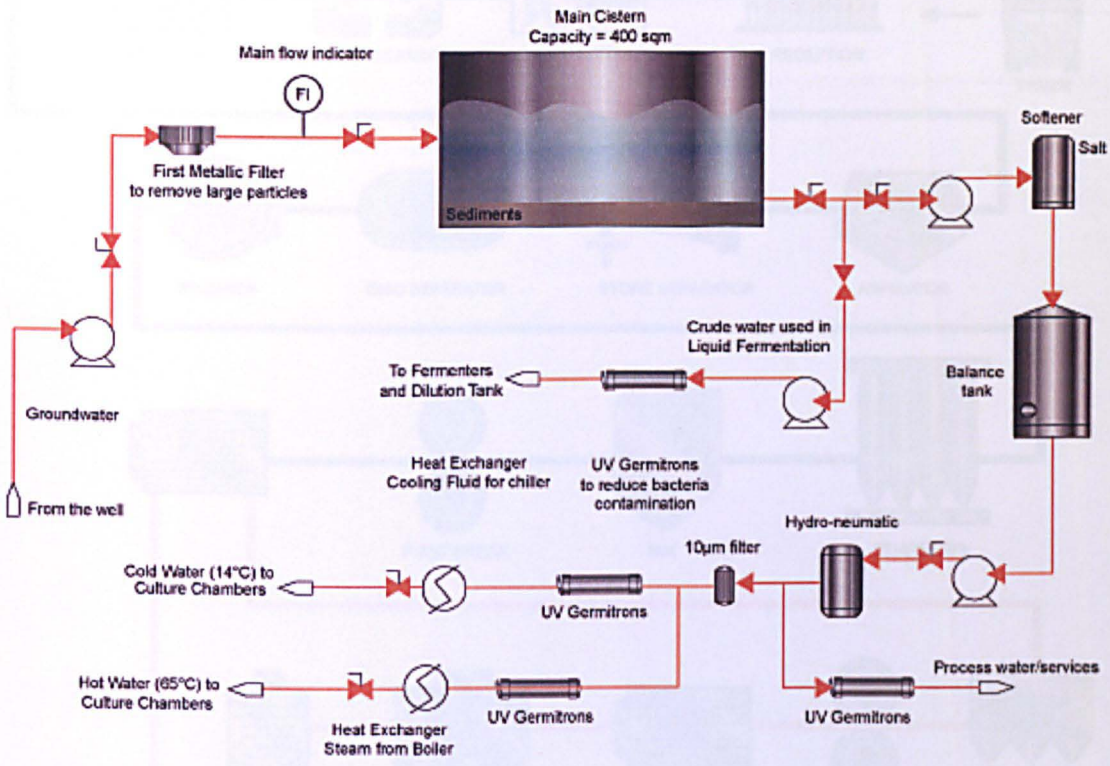


Figure 2.18 Water system in Serdán plant.

2.4 Wheat Bran Manufacture

Wheat bran is the outer layer of the whole grain of wheat and this layer is removed during milling and is sold in bulk as a by-product. This papery brown coating is rich in fibre and nutrients and expands as it absorbs large amounts of water. Wheat bran for human intake is different from the regular wheat bran in respect to the particle size distribution. Wheat bran for human intake is sieved and most of the flakes are within the large range (i.e., particles higher than 1400 microns) with more than 75% of the particles in this range, while standard feed grade wheat bran only has about up to 20% of the particles in this size range. A typical process for the production of wheat bran is seen in Figure 2.19.

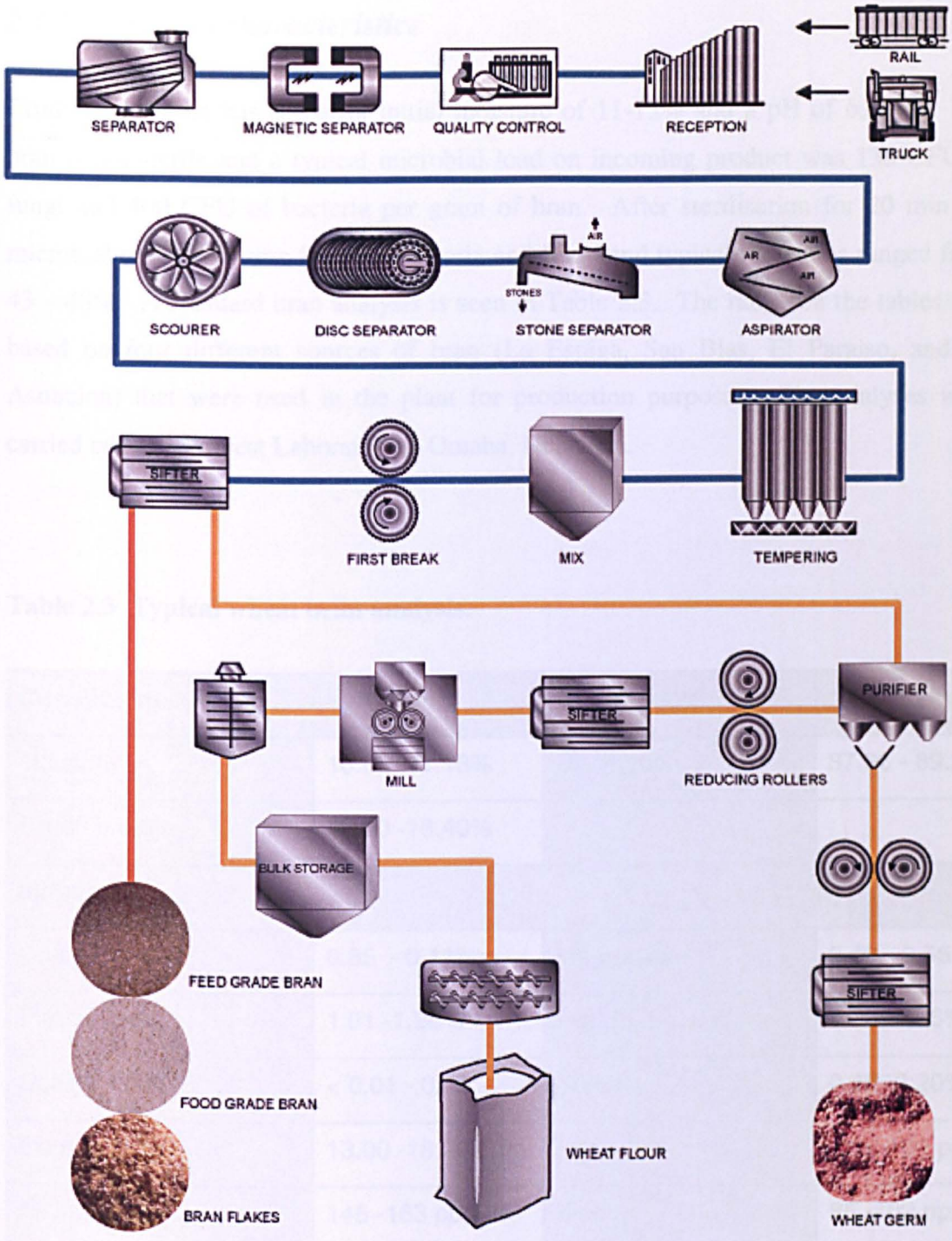


Figure 2.19 Process flow chart of flour milling and production of wheat bran (adapted from a flow chart from the North American Millers' Association, Washington, DC and Van Der Borght *et al.*, 2005).

2.4.1 *Substrate characteristics*

Crude wheat bran has a typical initial moisture of 11-15% and a pH of 6.2-6.5. The bran is not sterile and a typical microbial load on incoming product was 130 CFU of fungi and 400 CFU of bacteria per gram of bran. After sterilisation for 20 min the microbial count was zero for both bacteria and fungi and typical moistures ranged from 43 – 48%. A standard bran analysis is seen in Table 2.3. The range on the tables was based on four different sources of bran (La Espiga, San Blas, El Paraiso, and La Asuncion) that were used in the plant for production purposes. The analyses were carried out by Midwest Laboratories, Omaha, Nebraska.

Table 2.3 Typical wheat bran analysis.

Constituent	**% or ppm**	**Constituent**	**% or ppm**
Moisture	10.80 -11.13%	Dry matter	87.96 - 89.20%
Crude protein	16.20 -18.40%		
Minerals			
Calcium	0.85 - 0.11%	Magnesium	0.40 - 0.55 %
Phosphorous	1.01 -1.28%	Potassium	1.23 -1.36%
Sodium	< 0.01 -.02%	Sulfur	0.18 -0.20%
Copper	13.00 -18.00 ppm	Iron	123 -161 ppm
Manganese	145 -163 ppm	Zinc	85 -103 ppm
Toxins			
Deoxynivalenol and 3 Acetyl DON,	< 0.5 ppm	Aflatoxin B2, G1, G2	< 5 ppm
Zearalenone	< 0.5 ppm	T-2 Toxin	< 0.5 ppm

2.4.2 *Sterilisation equipment and process*

Wheat bran was sterilised in a steriliser (Fig. 2.20) manufactured by Littleford Day (Florence, Kentucky). For every sterilisation cycle, the equipment was first cleaned by utilising the CIP system. Wheat bran (480kg) was loaded via conveyer into the steriliser rotating at 52 RPM velocity. Once the bran was loaded, the steriliser increased speed to 93 RPM to induced better mixing and homogeneity. The steam was supplied to the jacket and when the temperature reached 70°C, steam was allowed to enter the chamber from both the top and bottom. The temperature was allowed to reach 118°C and the bran was held at this temperature for 20 min at 93 RPM. Pressure was then released and the system was cooled by opening the cool water valves until the bran was cooled to 40°C. Using sterile gloves, a representative sample was removed for laboratory analysis to confirm sterility of the bran and for comparative fermentations in the laboratory.

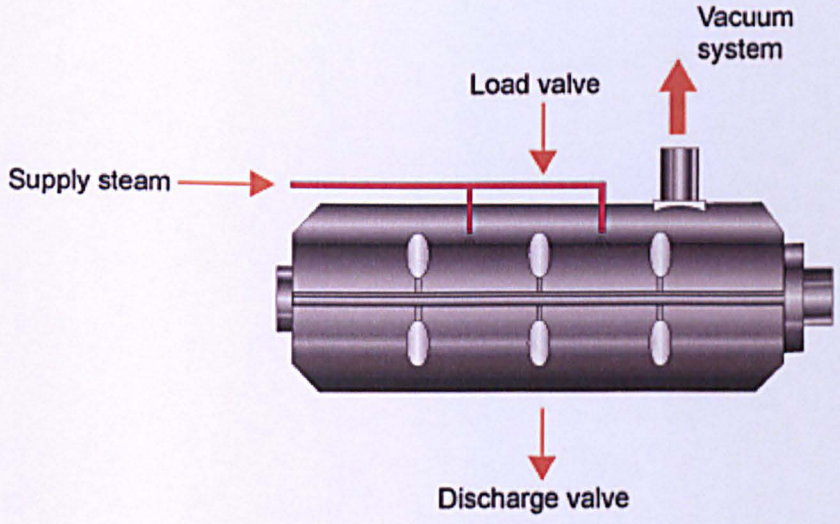


Figure 2.20 Schematic of the bran sterilising equipment.

To obtain a standard curve for the procedure, the temperature was monitored every two min on six sterilisation runs. Figure 2.21 shows a representative temperature profile for the sterilisation procedure. This temperature profile was consistent for all of the experiments reported in this study unless mentioned otherwise.

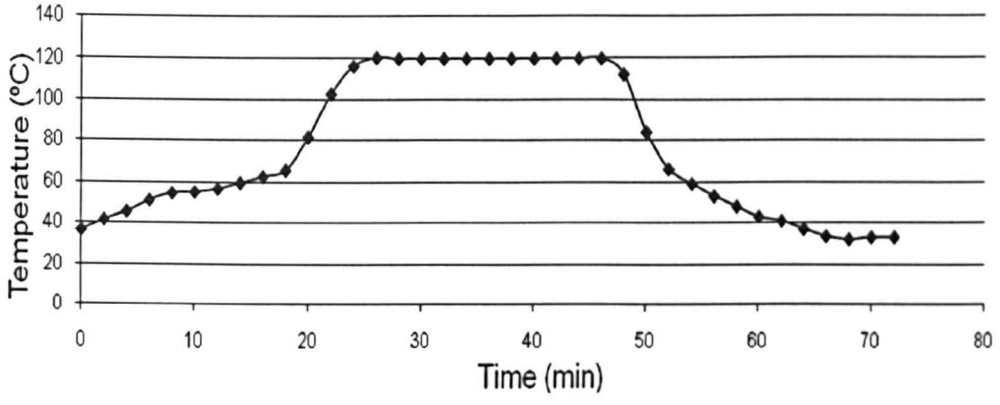


Figure 2.21 A representative temperature profile for a typical wheat bran sterilisation procedure.

2.4.3 Inoculation of wheat bran

Wheat bran was inoculated by transferring liquid containing the culture organism directly into the steriliser containing the cooled sterilised bran. The transfer line to the steriliser from the inoculum dilution tank was sanitised using steam sterilisation lasting 1 h. Five litres of inoculum was passed through the line to cool it and was drained to waste. A flow meter (Magflow, Danfoss) was used to measure the transfer of the inoculum. The steriliser was supplied with sterile air and kept at a manometric pressure of 0.34 until it was time to inoculate, at which time the air supply was stopped and the liquid inoculum (see Section 2.6.2 for growth of starter culture details) was transferred into the rotating drum (45 RPM). The transfer lines were purged with sterile air and the inoculated wheat bran was mixed for 8 min to ensure even distribution of inoculum before transfer to individual koji growth trays.

2.4.4 Buffering capacity of bran

To control the pH in wheat bran SSF is extremely difficult, but the substrate does offer some buffering capacity that helps maintain the pH of the substrate temporarily once the

organism starts to produce metabolic by-products.

A number of experiments were carried out to measure the extent of the buffering capacity of the plant wheat bran and also to determine how much acid would be required to lower the pH of the bran. Titration curves were prepared using both 5% (v/v) formic acid and 5% (v/v) phosphoric acid. The acids were added in 2 mL increments. It was shown that 33.6 L of 5% formic acid (Fig. 2.22) was needed to lower the pH of wheat bran for a regular tray filling (i.e., 480kg of bran) from a pH of 6.6 to a pH 5.0 and with 5% phosphoric acid, 48 L was required (Fig. 2.23).

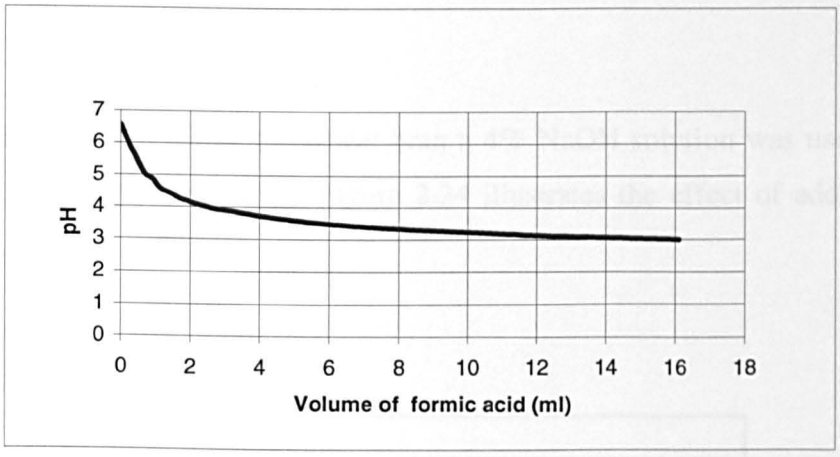


Figure 2.22 Effect of addition of 5% formic acid in 2 mL increments on the pH of the wheat bran substrate.

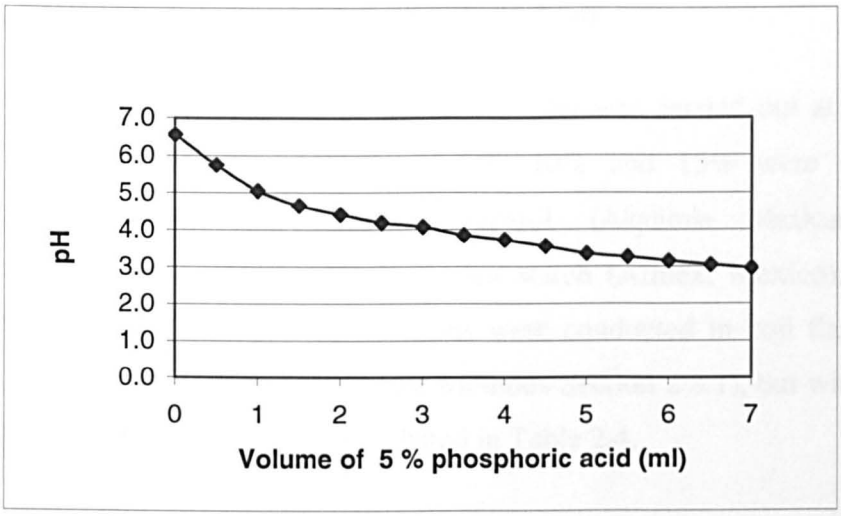


Figure 2.23 Effect of addition of 5% phosphoric acid in 2 mL increments on the pH of the wheat bran substrate.

To raise the pH of the wheat bran a 4% NaOH solution was used and it was added in increments of 0.5 mL. Figure 2.24 illustrates the effect of addition of caustic on the wheat bran pH.

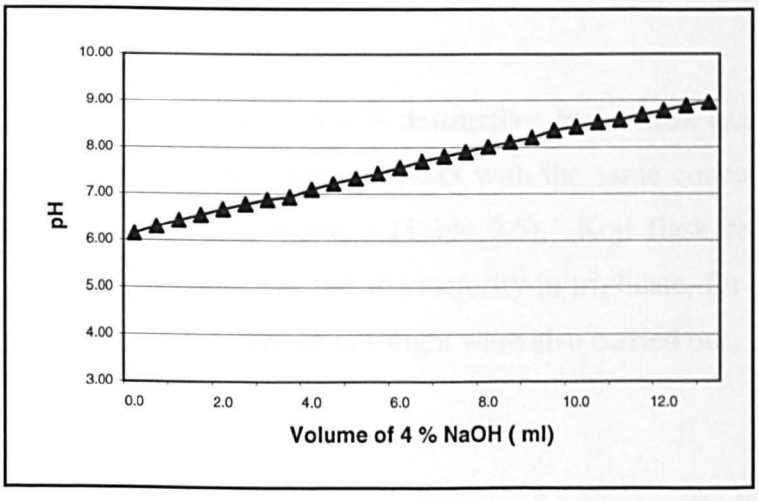


Figure 2.24 Effect of addition of 4% NaOH in 0.5 mL increments on the pH of the wheat bran substrate.

2.5 Other Substrates Added To Wheat Bran

Nutrient supplementation of the wheat bran was carried out at different levels. The following substrate additions at 5%, 10% and 15% were used for wheat bran supplementation experiments: glycerol (Alquimia Mexicana, Mexico), gluten (Comercializadora Veyco, Mexico), corn starch (Almex, Mexico), and soya bean meal (Tehuacan, Mexico). Fermentations were conducted in koji flasks at 30°C using the standard koji flask technique (see Methods Section 2.8.1), but with the incorporation of the four substrates at the levels listed in Table 2.4.

Table 2.4 Level of additions to wheat bran for koji flask fermentations.

Wheatbran with no additions	Glycerol	Soya	Corn Starch	Gluten
100%	5%	5%	5%	5%
100%	10%	10%	10%	10%
100%	15%	15%	15%	15%

Since the sampling process is destructive, and a flask can only be sampled once, it was necessary to set up multiple flasks with the same concentration so that they could be sampled on day 3, 4 and 5 (Table 2.5). Koji flask fermentations were analysed at minimum in duplicate, and the majority in triplicate, for phytase activity. Analyses for pH, moisture and change in weight were also carried out.

Table 2.5 Number of koji flasks for experiments.

	Wheat Bran Control	Replicates	Substrates	Treatments	Treatment Total	Total Koji Flasks
Initial	1	1	4	3	12	13
Day 3	3	3	4	3	36	39
Day 4	3	3	4	3	36	39
Day 5	1	1	4	3	12	13
Total Koji Flasks						104

Mineral supplementation was also carried out, focusing on zinc levels using zinc acetate and manganese levels using manganese acetate, at different levels as described later in this document.

2.6 Liquid Fermentation Stage

2.6.1 Inoculation from slope to liquid

Fungal cultures were cultivated on Difco Potato Dextrose Agar (PDA) plates to confirm purity and single colony isolates were transferred from the plates to PDA slants. Batches of 120 slants were made at one time. Slants were incubated at 30°C for two weeks in order to allow the culture to mature. They were then used for a maximum of fifteen days in production. Five slants from each batch were preselected in order to perform a quality control standard for the entire batch. The preselected slants entered the S2 stage and were inoculated into koji flasks in order for their activity to be assayed after a four day fermentation. If the activity was high enough, the batch was approved for production. Slants were then selected based on the best morphology. Good levels of sporulation were desired with no visual yellowing of the strain on the slope. The yellow colour was due to a spontaneous gene mutation and those colonies isolated with this mutation resulted in lower enzymatic activity. Approved slants were individually

inoculated into a 200 mL flask containing liquid medium. The flasks were shaken (200 rpm) at 30°C for three days. This stage of the cultivation is called S2 throughout this study. The liquid S2 medium consisted of the following: 0.5% (w/v) Corn Products International Starch (Veyco, Mexico City, Mexico); 1.8% (w/v) Difco yeast extract; 0.05% (w/v) KCl; 0.15% (w/v) $MgSO_4 \cdot 7H_2O$; 0.10% (w/v) KH_2PO_4; 0.2% (w/v) $CaCl_2$; 2.0% (w/v) production wheat bran and distilled water.

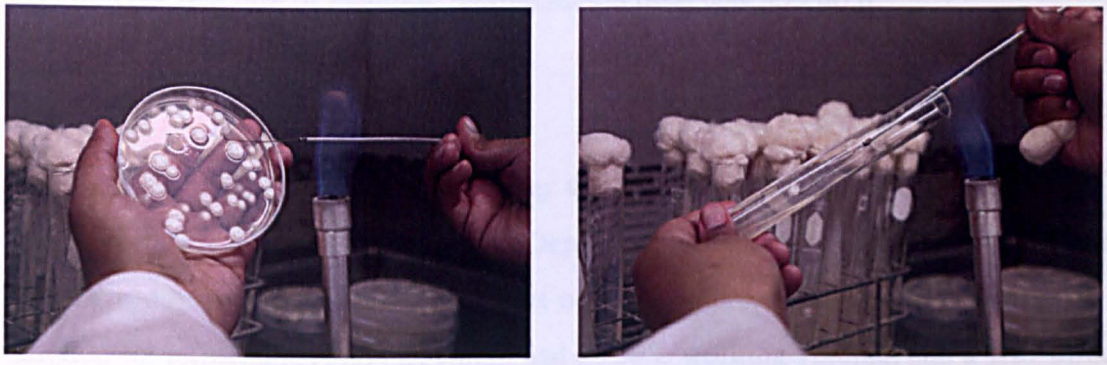


Figure 2.25 Stage 1 Propagation – plate to slope procedures.

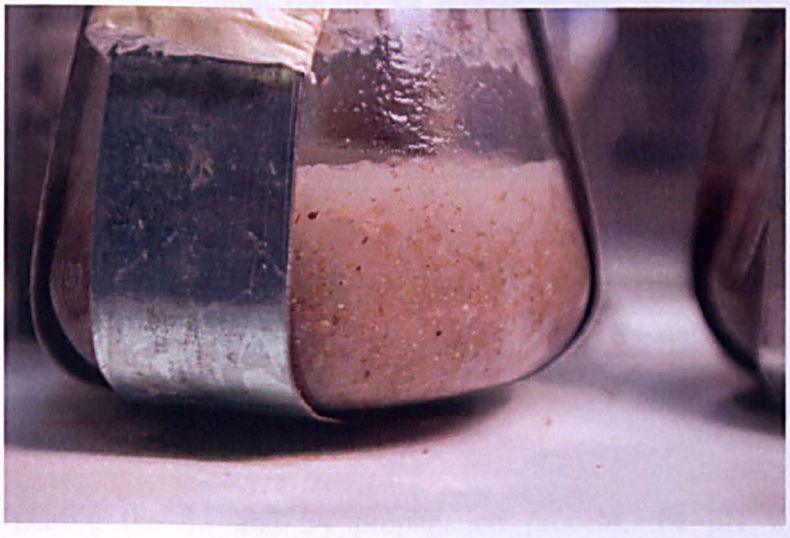


Figure 2.26 S2 flask (200 mL of medium in a 500 mL flask).

One S2 flask (200 mL of medium in a 500 mL flask) (Fig. 2.26) was used to inoculate one S3 fermenter (i.e. the second propagation step) at an initial dilution of 1:200.

2.6.2 Liquid medium preparation and scale up (S2, S3 and S4)

The liquid medium for the S3 fermenter (30 L working volume) was as follows: 2.8% (w/v) Corn Products International Starch; 1.8% (w/v) Difco yeast extract; 0.05% (w/v) KCl; 0.15% (w/v) $MgSO_4 \cdot 7H_2O$; 0.1% (w/v) KH_2PO_4; 0.2% (w/v) $CaCl_2 \cdot H_2O$; 2.0% (w/v) production wheat bran; 0.05% (w/v) glucose and antifoam at 0.02% (Midland PCS 4001, Ecolab, St. Paul, MN) and well water. The fermenter was operated 30°C, a manometric pressure of 0.68, agitation was 225 rpm, and oxygen was introduced at 1.0 L_{air}/ min per one litre of medium. Fermentation time was 30 - 40 h.

Biomass produced from the S3 fermenter was visually inspected and transferred to the S4 medium for further propagation. Depending on the S4 fermenter size, different inoculum dilutions were used at the start of biomass propagation in S4, namely a 1:12 dilution for fermenters A and B, which had a 350 L working volume, and a 1:27 dilution for the larger fermenters, C and D, which had an 800 L working volume.

The liquid medium for the S4 fermenter was as follows: 2.8% (w/v) Corn Products International starch; 0.9% (w/v) yeast extract Difco; 0.1% (w/v); (w/v) KCl; .075% (w/v) $MgSO_4 \cdot 7H_2O$; 0.05% (w/v) KH_2PO_4; 0.1% (w/v) $CaCl_2 \cdot H_2O$; 0.075% (w/v); 3.0% (w/v) wheat bran and antifoam at 0.02% (Midland PCS 4001, Ecolab, St. Paul, Minnesota) and well water. The fermenter was operated at 35°C, with a manometric pressure of 0.68, agitation was 175 rpm, and oxygen was introduced at 0.5 L_{air}/ min per one litre of medium. Propagation time for the S4 stage was 12 – 16 h.

During the S3 and S4 propagation process, the pH was not controlled, but pH measurement was monitored as an indicator of possible fungal and/or bacterial contamination.

2.7 Control of Contamination

2.7.1 Sanitisation of equipment, rooms, trays and trolleys

2.7.1.1 Sanitisation of chambers

The chambers were cleaned of bran debris and all areas were rinsed with high pressure cold water (ceiling, wall, floors, hopper, and conveyor). An alkaline foam sanitiser (Klenzade 215, Ecolab St. Paul, Minnesota) at a concentration of 0.8% (v/v) was left on all contact surfaces for 10 min and then washed off with cold water. This was followed by the use of the liquid ammonium quaternary salt disinfectant, Sterbac (Ecolab, Ecolab St. Paul, Minnesota) (0.39% v/v), which was left on all contact surfaces for 10 min and then washed off with cold water. The final step in the sanitation process for the chambers was the use of an 8% (w/v) solution of diammonium propionate (Moldzap, Alltech, Lexington, Kentucky). This product was only used in areas where there was no direct contact with the koji and where there was no stainless steel equipment.

2.7.1.2 Sanitisation of tray filling area

A mobile surface washer (MSW/456, ClearWater Tech, Luis Obispo, California), equipped with ozone-enriched water was used to disinfect the walls, floors and drains in the tray filling area. This method of sanitising was more effective than chlorine. It also reduced water and energy consumption and left no harmful residual chemicals.

2.7.1.3 Sanitisation of trolleys

As the trolleys arrived from the unloading section to the wash area, each trolley initially was washed from top to bottom, including wheels, using a long hard bristle hand brush. The cleaning solution was a chlorine/detergent (lineal anionic surfactant) solution (CLOROLIMP, Mexico City, Mexico). The brushed trolleys were moved to the wash area where high pressure water (KARCHER Model HD 1000C, Chantilly, Virginia) was sprayed on all parts of trolleys. The direction was from top to bottom and then repeated from bottom to top. After the high pressure water wash, each trolley was

washed again using pressured water from a tank containing a benzyl solution (8 g/ L benzyl conium chloride) and sprayed from top to bottom. The benzyl washed trolleys were moved into the sterile zone, where there was a ramp (pool) containing chlorinated water. The trolleys were placed so that the wheels were submerged. After 20 min they were moved into the sterile production area for the tray filling process.

2.7.1.4 Sanitisation of trays and lids

An in-house adapted commercial tray washer, that uses hot water and steam, was employed to sanitise the trays. The temperature of the washer was 70°C and detergent (ROMA, Mexico City, Mexico) was used. Trays and lids arrived to the wash area via conveyers. As the trays and lids moved along the washer, at different points there were spray nozzles that sprayed detergent water, as well as brushes that scraped the surface of the trays/lids. The length of the detergent washing stage lasted 5 min, as did the rinsing. The tray/lids from the tray washer were stacked vertically to drain the water and placed on platforms that could hold 180 to 200 trays/lids. The platforms, in batches of three, were moved into the autoclave for sterilisation at 121° C for 1 h. As soon as the sterilisation process was complete, the sterile trays and lids were moved to a tray filling area, cooled and used for production.

2.7.1.5 Sanitisation of conveyor belts and UV (sterilisation)

Once the koji was sterilised it was dropped onto a conveyor belt for distribution to the koji trays. To assist in keeping contamination minimal in this belt area, UV lights are used around the belt (Fig. 2.27).

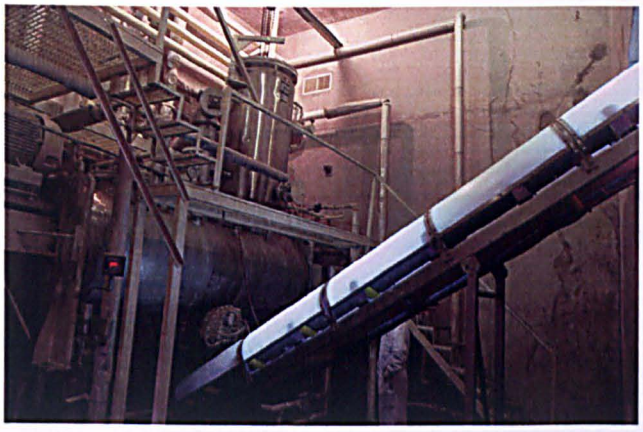


Figure 2.27 Bran tray loading conveyor belt with UV lights.

2.7.2 Process water

Water is used extensively in the plant, particularly in the cooling process for the fermentations. The process is very clean and very little effluent is created. Sterility of the water is a critical parameter. Ultraviolet light is used to ensure that the water is clean as it enters into the process areas.

Process water samples were collected in sterile Erlenmeyer flasks and 1 mL aliquots mixed for use in pour plates using Standard Agar (CMA, BD Bioxon, Becton Dickinson, Mexico). The plates were incubated for 48 h at 37°C to monitor that no contaminating organisms were present.

2.7.3 Operator access

Personnel working in the inoculation, tray filling and fermentation areas of the plant are all required to change from their street clothes into white sterile uniforms before entering the restricted areas. Restricted areas are defined as these areas where contamination must be kept to a minimum and the areas are accessed only through a change room. Outside clothing is left in the change room and restricted areas are entered only by personnel wearing sterile outer clothing, boots and face masks (see Fig.

2.28).

Figure 2.28 Typical clothing worn when accessing restricted fermentation areas.

2.7.4 Equipment and surfaces

An area of 100 cm^2 was swabbed using a cotton swab moistened with sterile saline solution. The swab was rinsed into 10 mL of 0.1% NaCl solution, diluted as required, and the dilutions plated onto CMA Standard Agar.

2.7.5 Air handling

To test the air in chambers, open plates of CMA Standard Agar and Difco PDA medium were left open to the air for 5 min. The exposed plates were incubated for two days at 37°C.

2.7.6 In process microbial analysis

Aliquots of the product (20 g) were placed into 180 mL of 0.1% sterile NaCl solution in a 200 mL Erlenmeyer flask. The flask was shaken for 1 h at 200 RPM, allowed to stand

for 3 min, and a 1 mL sample was removed, diluted as required (usually ~10^6) and plated onto a CMA Standard Agar plate and incubated for 48 h at 37°C.

Difco Potato Dextrose Agar 213400 (Becton Dickinson and Company, New Jersey) was used for culturing and enumerating yeast and moulds. A 23.5 g aliquot of the medium was suspended in 1 L of dH_2O water, mixed, heated with agitation, boiled for 1 min to dissolve the powder and autoclaved at 121°C for 15 min. Difco Plate Count Agar 247940 (Becton Dickinson and Company, New Jersey) was used for culturing and enumeration of bacteria. A 45 g aliquot of the medium was suspended in 1 L of dH_2O water, mixed, heated with agitation, boiled for 1 min to dissolve the powder and autoclaved at 121°C for 15 min.

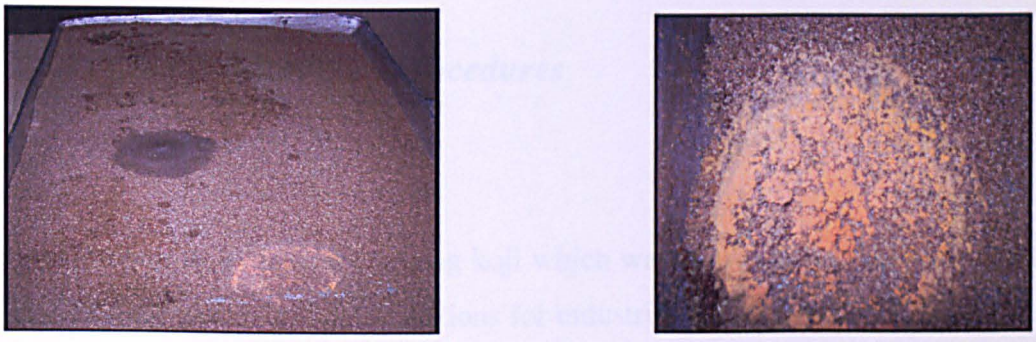
Samples for analysis were diluted to a number where fungi (5-20 colonies per plate) and bacteria (20-200 colonies per plate) could be counted. Serial dilutions were performed using sterile water and diluted samples were plated in triplicate using pour plates. Plate count agar plates were inverted and incubated at 35 ± 2°C for 48 h. The PDA plates were inverted and incubated at 20 ± 2°C for 48 h. Results were reported as colony forming units (CFU)/g.

2.7.7 Common contaminants

The organisms most commonly detected as bacterial contaminants in the chambers were sent to the University of Nuevo Leon in Mexico for characterisation using the BIOLOG GP2 microplate (Biolog, Inc., Hayward, CA.). The bacteria were identified as *Bacillus subtilis, Bacillus megaterium, Micrococcus luteus, Cellulomonas hominis, Alloiococcus otitis, Cellulosimicrobium and Paenibacillus polymyxa* (Appendix A). All bacteria identified were ones that one would expect to encounter in the air and from a plant environment. There were also two troublesome fungi that could be seen growing on the trays and these were very invasive and problematic in terms of growth on the koji trays. One fungus was orange in colour and one was grey/black in colour and both appeared

very different in appearance from the plant production fungal strains. These fungi were also sent out for identification (Microcheck–Microbial Analysis Laboratory, Northfield, Vermont) and identified as *Monilia cinerea var americana* (red bread mould/fruit mould) and as *R. stolonifer* (common black bread mould).

Figure 2.29 Part A illustrates a tray contaminated with the red fungus *Monilia cinerea var americana* and the black fungus *R. stolonifer*. When these fungi cover the surface of the bran they inhibit the growth of the production organisms resulting in poor enzyme yields per tray.



A B

Figure 2.29 (A) Production koji tray showing growth of contaminating black (*R. stolonifer*) and orange fungus (*Monilia cinerea var americana*) growing on the surface of the wheat bran after three days in the fermentation chamber. (B) Higher magnification of orange fungus covering the surface of the bran with fluffy mycelia visible.

2.8 SSF Parameters

Three key parameters that affect the fermentation are pH, moisture and heat removal.

2.8.1 Koji flask procedures

Koji flask fermentations were incubated using laboratory incubators with a relative

humidity of 60% (Stericult 200 Mod-3194, Scientific, Marietta, Ohio, USA and Model B-5061, Heraeus Instruments, Hanau, Germany) or in a 3.0 x 3.0 x 3.2 m special incubation room that was constructed for this purpose. In this incubation room, the temperature fluctuated between 30-32°C and the humidity between 65–76%. This incubation room was used as an attempt to mimic the large production fermentation chambers. In the large production chambers the koji growth takes place on trays covered with lids. This room was used as a chamber substitute for the koji flask experiments, as it was similar to the plant in that the temperature regulation spanned a higher range, as did the regulation of humidity. A large numbers of flasks could be placed in the room at one time, whereas with the laboratory incubators the number of samples that could be handled was limited.

2.8.2 Chamber and tray procedures

Two 11 litre SSF trays, containing koji which was grown under standard conditions of growth, time and inoculum conditions for industrial phytase production were selected at day 2, 3 and 4 of incubation from the trolley locations indicated in Figure 2.30 and Table 2.6 after incubation in a commercial scale incubation chamber (See Section 2.13.4 regarding methodology for selecting the tray locations within the chamber).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1															
2	1T1	1T3	1T5	2T1	2T3	2T5	3T1	3T3	3T5	4T1	4T3	4T5	5T1	5T3	5T5
3	1T2	1T4	1T6	2T2	2T4	2T6	3T2	3T4	3T6	4T2	4T4	4T6	5T2	5T4	5T6
4															

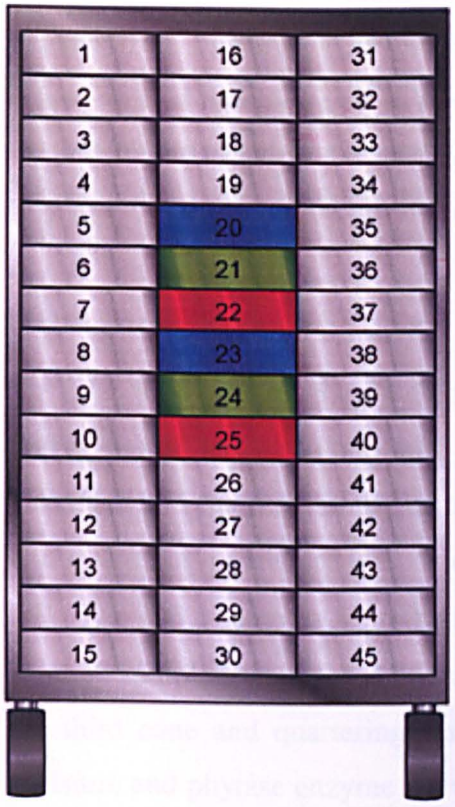
Note: The yellow colour indicates the trolleys that were sampled to examine chamber variance.

Figure 2.30 Trolley locations in commercial scale incubation chamber 4.

Table 2.6 Schematic to identify locations of trays to be sampled for chamber variance

	Chamber		Tray Level Address		
Sample	Row	Column	Day 2	Day3	Day 4
1T1	2	1	20, 23	21, 24	22, 25
1T2	3	1	20, 23	21, 24	22, 25
1T5	2	3	20, 23	21, 24	22, 25
2T1	2	4	20, 23	21, 24	22, 25
2T6	3	6	20, 23	21, 24	22, 25
3T2	3	7	20, 23	21, 24	22, 25
3T5	2	9	20, 23	21, 24	22, 25
4T1	2	10	20, 23	21, 24	22, 25
4T6	3	12	20, 23	21, 24	22, 25
5T2	3	13	20, 23	21, 24	22, 25
5T5	2	15	20, 23	21, 24	22, 25
5T6	3	15	20, 23	21, 24	22, 25

Note: Colour indicates sampling days and locations of trays with blue samples indicating day 2, green indicating day 3 and red indicating day 4.



Note:

Day 2 samples indicated by the colour blue

Day 3 samples indicated by the colour green

Day 4 samples indicated by the colour red

Figure 2.31 Schematic of trolley that holds koji trays, with trays selected for analysis indicated corresponding to the sampling days in Table 2.6.

The trays were removed from the middle of the trolley on day 2, 3 and 4 of incubation as indicated in Figure 2.31 and the content of each tray (~2.8kg) was emptied into a 5 litre plastic mixing bag.

The exact weight of the wet koji in each tray was recorded and the percentage moisture was determined before fluidised bed drying. After the fluidised bed drying process, the weight and residual moisture of the dried koji was recorded.

The contents of each of the four trays were subjected to fluidised bed drying with a drying time of ~15 min per batch. To dry the contents of one tray required three to four

batches due to the small size of the laboratory dryer (Aeromatic AG Model STP RZ 02, Muttenz, Switzerland). After the batches were dried to ~10% (w/w) moisture, all batches from one tray were placed into a fresh 5kg plastic mixing bag, the bag was closed with as much air in it as possible and rotated 20 times to the right and 20 times to the left to ensure adequate mixing. The bag contents were then placed onto a mixing tray and the cone and quarter method applied.

To cone and quarter, the ~1.5kg dried sample was piled into a cone shape with a flattened top, and the cone was divided into quarters. The opposite quarters were discarded and the remaining quarters mixed together to form a second cone. The cone and quartering procedure was repeated three times, until only a sample of ~100 g was left. The 100 g samples from the first and second cone and quarter were stored at 21°C in small sample bags, in case additional analyses were required. The 100 g sample from the third cone and quartering was placed into a small sample bag and analysed for moisture and phytase enzyme activity (in triplicate) within 48 h for each of the 72 trays sampled. Analysis of variance was conducted using nested design as described in Section 2.12.

All sampling and analyses were labelled with the name of the operator so that possible changes due to operator/analyst variation could be identified. Temperature in the chambers was monitored.

2.8.3 Control of pH

To raise the pH of the wheat bran on a production scale, the following process was employed. A 1 N solution of NaOH was placed into a stainless steel reactor, sterilised for 30 min, cooled and transferred from the vessel to the bran steriliser using a sterile transfer line and flow meter. The sterilised bran and the NaOH solution were mixed for 10 min at 94 rpm and the pH measured to ensure that the desired pH had been reached.

2.8.4 *Heat removal and temperature control*

As discussed, one of the most critical parameters in the fermentation is temperature control. The system used RTDs, which controlled the hot and cold water supply to keep the temperature as close to the fermentation temperature profile as possible. The RTDs were regularly tested and compared to mercury thermometer measurements. Figure 2.32 is a typical temperature profile over 100 h with a desired set temperature of 32°C.

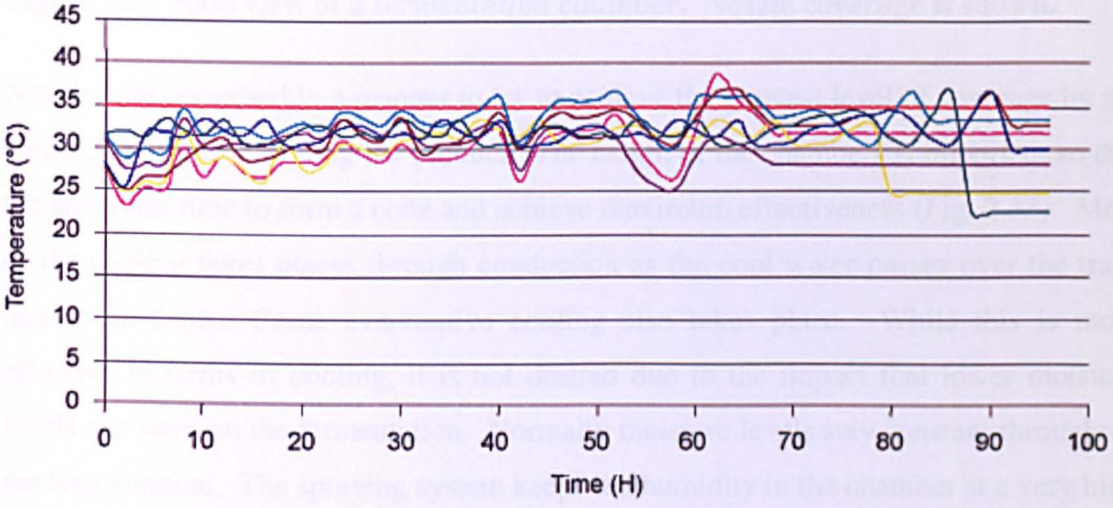


Figure 2.32 Example of temperature profile of a commercial *A. oryzae* fermentation over 96 h.

Water systems were used in the SSF chambers as the primary heat removal and temperature control system.

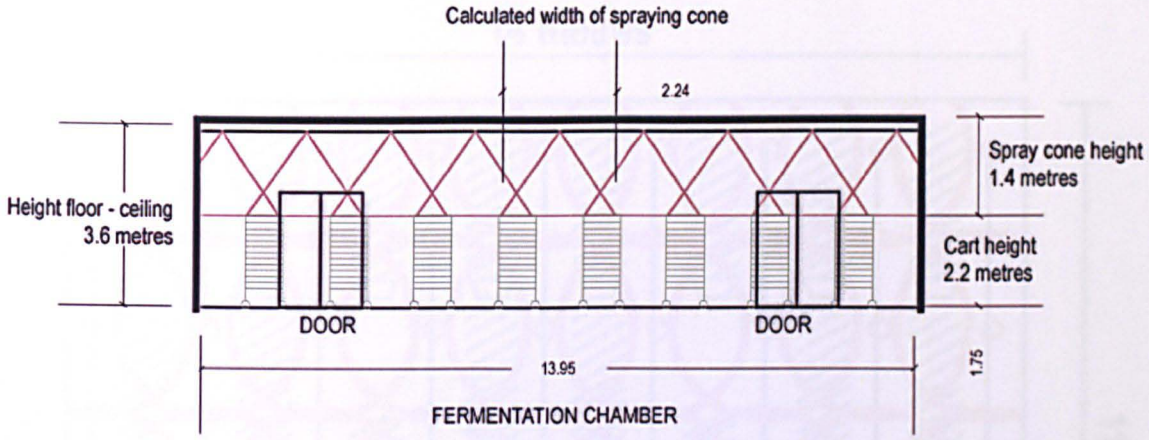


Figure 2.33 Side view of a fermentation chamber. Nozzle coverage is shown.

Nozzles are organised in a manner to try to achieve the greatest level of coverage by the sprays, but without wetting the product. The height of the chambers is important so that the spray has time to form a cone and achieve maximum effectiveness (Fig. 2.33). Most of the cooling takes places through conduction as the cool water passes over the trays and cools them. Some evaporative cooling also takes place. While this is more effective in terms of cooling, it is not desired due to the impact that lower moisture levels can have on the fermentation. Normally moisture levels stay constant throughout the fermentation. The spraying system keeps the humidity in the chamber at a very high level. Figure 2.34 details the coverage by the spray nozzles where the green lines illustrate the pipes, centre points are the spray nozzles and coverage is marked with red overlapping circles.

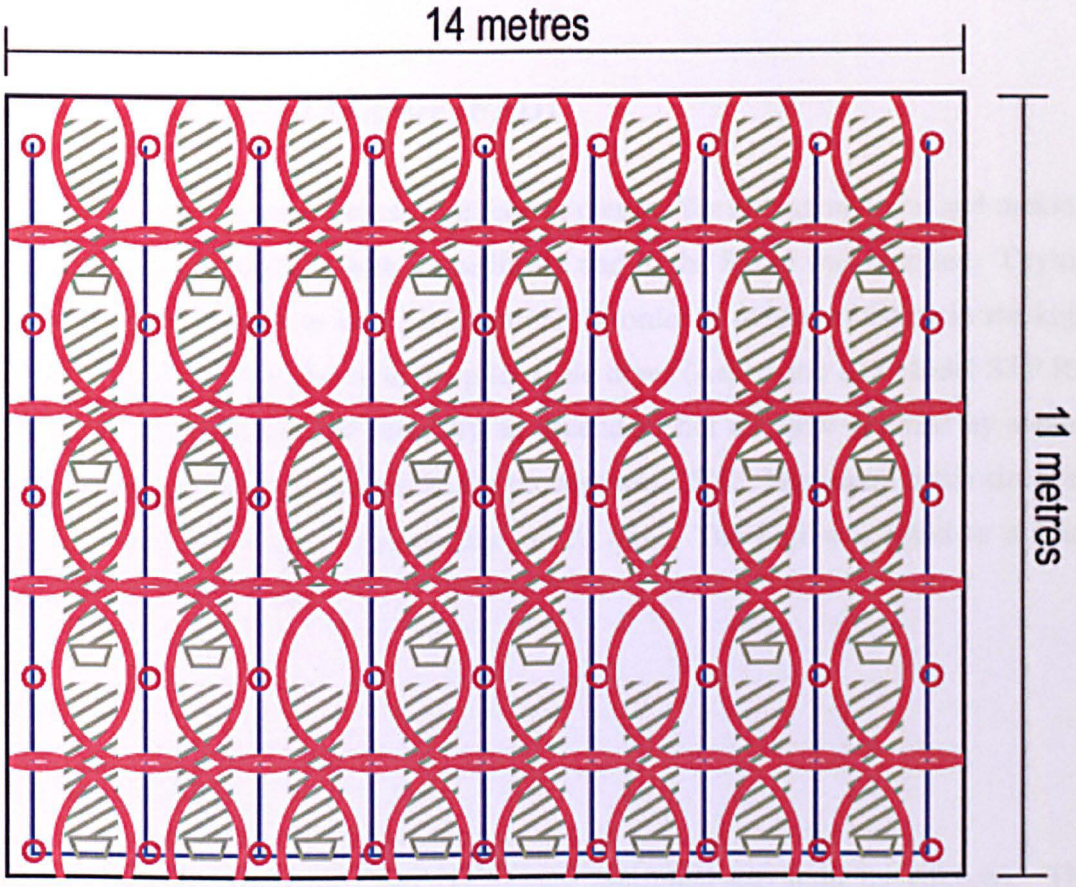


Figure 2.34 Top view of a fermentation chamber.

To achieve the temperature profile desired, it was necessary to spray either cold or hot water at certain times of the fermentation. This water is important in order to keep the levels of relative humidity that are also required to obtain a satisfactory fermentation. The general conditions in the chamber for fermentation are 95% relative humidity and a temperature in the product tray of 30°C.

2.9 Drying and Final Product

Drying the product allows analysis to be carried out later, protects the enzymes in the product and also minimises microbial contamination. It is achieved through the use of air systems that drive off moisture. Typically moisture levels should be below 12%.

2.9.1 *Pilot scale fluid bed dryer (FBD)*

For smaller samples in the process, an effective method for storing material and making it easier to carry out analysis both immediately and in the future was required. Drying in a smaller dryer seemed to be a good option. In order to reduce moisture in the koji, 800 g of the product was placed in the pilot scale dryer (Aeromatic AG Model STP RZ 02, Muttenz, Switzerland) and dried by an ascendant hot air flow warmed by steam. The maximum temperature allowed in the process was 75°C. Maximum batch size was 400 g (dry product) with a process time of 20 min. The koji was dried to a final moisture level of 10 – 12%.

2.9.2 *Oven dryer*

Wet koji was dried on trays in an oven dryer (designed and built by Alltech). The incoming air was heated by a gas flame and a fan circulated the hot air in the oven. Each drying batch was capable of processing 12 koji trays. After loading all the trays, the dryer was activated and the incoming air allowed to reach 70°C. The hot air was circulated for 8 h, and every 2 h the koji on the individual trays was hand mixed to ensure uniform drying, to a final moisture level of 10 – 12%.

2.9.3 *Plant scale fluid bed dryer (FBD)*

In order to reduce moisture in the koji, the product was conveyed from the trays to a fluid bed dryer and dried by an ascendant hot air flow warmed by steam. The maximum temperature allowed in the process was 42°C. Maximum batch size was 350kg (dry product) with a process time of 50 min. The drying process reduced the moisture in the product from approximately 50% to 12%. For commercial product, after drying, the koji was milled and blended to obtain homogeneous enzyme activity and particle size per batch before packaging.

2.10.1 Trays (cone and quartering)

For sampling from the trays, due to the large quantity of koji on one tray (2.8kg) and the problem of the koji not growing consistently over the surface (i.e., higher growth in areas with better access to moisture, aeration and heat removal), it was necessary to cone and quarter the product from the trays. The sample was piled into a cone shape with a flattened top, and the cone divided into quarters. The opposite quarters were discarded and the remaining quarters mixed together to form a second cone. The process was repeated until the desired sample size, i.e. a sample the size of a hand ~75-100 g, was reached.

Unless noted otherwise, a minimum of three samples were taken after the cone and quartering process for analysis. Each of these samples was subjected to two separate dilutions and the results of the two dilutions were averaged (Fig. 2.35).

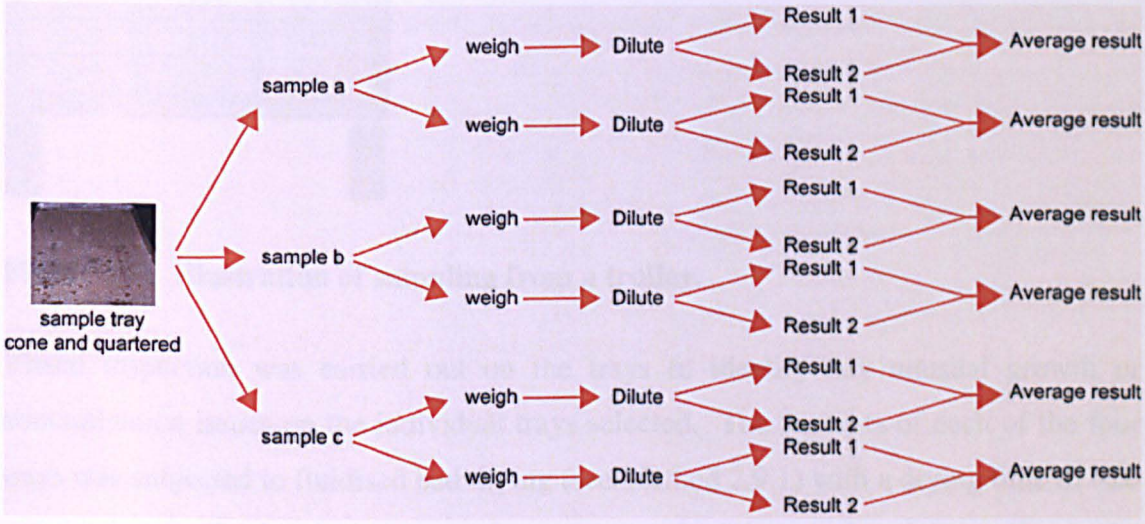


Figure 2.35 Illustration of sampling and analysis from a tray.

Sampling reproducibility and cone and quarter

Four 11 litre SSF trays containing koji, which had been grown under standard

conditions of growth, time and inoculum conditions for industrial phytase production for four days (see Section 2.8.2 for details) were selected from the middle of one trolley (see Figure 2.36). The content of each tray (~2.8kg) was emptied into a 5 litre plastic mixing bag. The weight of the wet koji in each tray was recorded and the percentage of moisture was determined before fluidised bed drying. After the fluidised bed drying process, the weight and residual moisture of the dried koji was recorded. This experiment was repeated one week later with the only change being better control of the fluidised bed drying process.

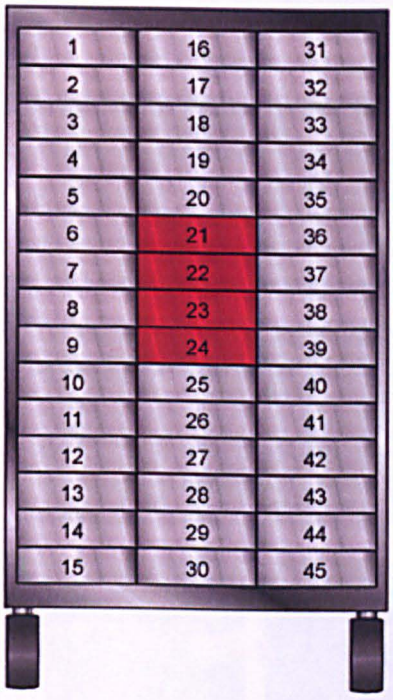


Figure 2.36 Illustration of sampling from a trolley.

Visual inspection was carried out on the trays to identify any unusual growth or contamination issues on the individual trays selected. The contents of each of the four trays was subjected to fluidised bed drying (see Method 2.9.1) with a drying time of ~20 min per batch. To dry the contents of one tray required three to four batches due to the small size of the laboratory dryer. After the batches were dried to ~10% (w/w) moisture, all batches from one tray were placed into a fresh 5kg plastic mixing bag, the bag was closed with as much air in it as possible and rotated 20 times to the right and 20 times to the left to ensure adequate mixing. The bag contents were then placed onto a mixing tray and the cone and quarter method applied.

To determine if additional cone and quartering influenced sampling and assay reproducibility, the following procedure illustrated in Figure 2.37 was followed. To cone and quarter, the ~1.5kg dried sample was piled into a cone shape with a flattened top, and the cone was divided into quarters. The opposite quarters were discarded and the remaining quarters mixed together to form a second cone. The cone and quartering procedure was repeated 10 times, each time until only a sample of ~100 g was left. The 100 g samples from the first to fifth sample and the tenth cone and quarter were placed into small sample bags and analysed for moisture and phytase enzyme activity (in triplicate) within 48 h, for each of the four trays sampled.

Figure 2.37 Photo of cone and quartering process.

All sampling and analyses were labelled with the name of the operator so that possible changes due to operator /analyst variation could be identified. Temperature in the chamber was recorded and monitored. The trays that were not sampled for the experiment were processed in batches of 480kg using a commercial size fluidised bed dryer. These batches were placed into large sacks and samples were taken from these sacks and moisture and phytase analyses conducted. In order to assure sampling and assay reproducibility, standard methods were tested.

2.10.2 *Flasks*

For the koji flask experiments, unless mentioned otherwise, experiments were conducted in triplicate with the contents of the flask being rehydrated and then dilutions made from this suspension. Each of these samples was subjected to two separate dilutions and the results of the two dilutions were averaged (Fig. 2.38).

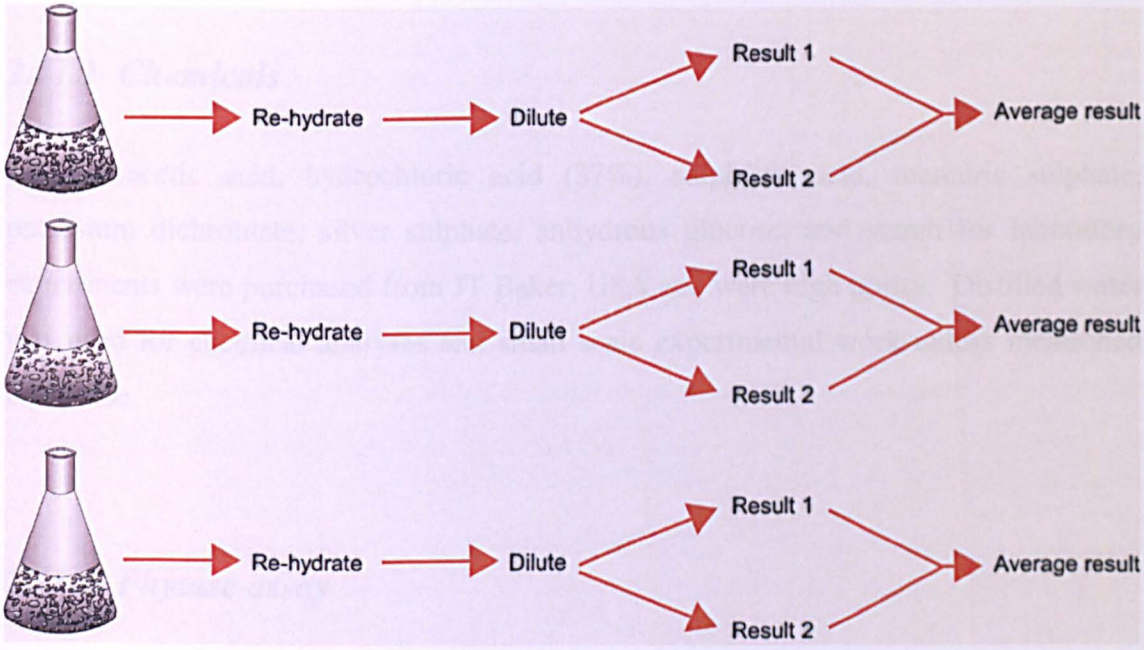


Figure 2.38 Illustration of analysis of koji flask.

2.10.3 *Sacks (sample rod)*

For post drying analysis, five samples of 250 g were taken from a 350kg bag by driving a grain probe (Seedburo Equipment, Chicago, Illinois) lance down through the middle of the sack and also down into the four corners. These five samples were then combined and mixed by hand and then using the cone and quarter method were further mixed and divided to obtain representative sized samples, which were placed in plastic bags and sent for analysis.

For final product analysis after processing through the grinding and blending system, larger samples are taken of 350 g. Five 350 g samples are taken from the 750kg final product sack using the same lancing method in the four corners of the sack and subsequently using the cone and quartering method again, in the same way as described above.

2.11 Standard Analyses

2.11.1 Chemicals

Trichloroacetic acid, hydrochloric acid (37%), sulphuric acid, mercuric sulphate, potassium dichromate, silver sulphate, anhydrous glucose, and starch for laboratory experiments were purchased from JT Baker, USA and were high purity. Distilled water was used for chemical analyses and small scale experimental work unless mentioned otherwise.

2.11.2 Phytase assay

Phytase activity was determined according to the procedure of Engelen *et al.* (1994). This assay is based on the enzymatic hydrolysis of sodium phytate under controlled conditions by measurement of the amount of ortho-phosphate released. Incubation is stopped by adding an ammonium molybdate/acetone reagent, which produces a coloured complex. The colour of the complex is measured at 380 nm. One SSF standard phytase unit (SPU) is defined as the amount of enzyme that will liberate 1 µmol of inorganic phosphate per minute at pH 5.5 and 37°C.

Sample Preparation

Adequately diluted samples (5 mL) were equilibrated to 37°C and mixed with a substrate solution consisting of 0.01 M phytic acid (Sigma catalogue number P8810, Sigma, St Louis, USA). This was prepared fresh daily in 0.2 M acetate buffer pH 5.5.

The mixture was incubated for 30 min at 37°C and then 2 mL of a cold stop solution (10 mM ammonium molybdate, 5 N sulphuric acid, and pure acetone, in a 1:1:2 ratio) was added and the mixture was vortexed. Citric acid (0.1 mL of a 1 M solution) was added and the mixture was again vortexed. Within 5 min of the addition of the citric acid, the absorbance, in a cell of 1 cm thickness and at a wavelength of λ_{380}, against water, was measured using the Hewlett Packard spectrophotometer which was described previously. This value was called the ODT (Optical Density Test).

Blank Preparation

An adequately diluted sample (0.5 mL) was mixed with 2 mL of the stop solution. Substrate solution (0.5 mL) was added to the mixture followed by the addition of 0.1 mL of 1 M citric acid. The absorbance of this sample (test blank) against water at λ_{380} was determined and this reading was called the OD_{TB} (Optical Density - test blank). The difference between the ODT and OD_{TB} was used to calculate the enzyme concentration from a standard calibration curve.

Standard Curve Preparation

The standard curve was prepared as follows: KH_2PO_4 (0.680 g) (P0662 - Sigma Chemical Co. Missouri) (previously dried in a desiccator with silica gel for 24 h) was dissolved in dH_2O to a final volume of 1000 mL and 1, 2, 3, and 4 mL aliquots of this solution were diluted with 50 mL dH_2O to obtain a standard dilution series with phosphate concentrations of 0.1, 0.2, 0.3 and 0.4 mmol/mL. Each standard solution (1 mL) was mixed with cold stop solution (2 mL) and allowed to stand for 30 sec followed by the addition of 0.1 mL 1 M citric acid and re-mixing. For the blank of the standard curve, dH_2O (1 mL) was used instead of the standard solution.

2.11.3 Extracellular protease assay

Protease activity was determined according to the procedure outlined in the Food Chemical Codex (1996). Five mL (0.2% (v/v) H2525-100G haemoglobin solution

(Sigma, St Louis, USA) was equilibrated to a temperature of 40°C. Adequately diluted sample (1 mL) and glycine/ HCl buffer (1 mL) (5 mM adjusted to pH 2.5) were added to the haemoglobin solution and incubated for 30 min at 40°C in a 20 L FE373 water-bath (Felisa, Jalisco, Mexico). To stop the reaction, 5 mL of 14% (w/w) trichloroacetic acid solution was added, and the solution was mixed and cooled for 15 min to bring it to room temperature. The solution was remixed and filtered through a #42 Whatman filter paper. The absorbance at λ_{275} was determined against a blank buffer (1 mL glycine/HCl instead of diluted sample), using a Hewlett Packard Model 8453 Spectrophotometer, (Hewlett Packard, Palo Alto), and the enzyme concentration calculated using the standard curve.

2.11.4 Extracellular cellulase assay

Cellulase activity was determined according to the procedure of Miller *et al.* (1960). One carboxymethyl cellulose (CMC) unit of activity liberates 1 μmol of reducing sugar (expressed as glucose equivalents) in 1 min under the conditions of the assay.

Test sample. The sample was diluted as required (using 0.05M sodium acetate buffer pH 4.8) and 1.0 mL of this dilution was equilibrated along with 1 mL of CMC substrate (carboxymethyl-cellulose, Sigma catalogue number C5678, St Louis, USA) to 50°C. One mL of equilibrated enzyme dilution was added to the CMC substrate and incubated at 50°C for 10 min. The reaction mixture was removed from the water bath and 3.0 mL of 3,5 - dinitrosalicylic acid solution (DNS) was added, mixed and placed in a boiling water bath for 5 min followed by cooling in an ice water bath.

Enzyme blank. CMC substrate solution (1.0 mL) was incubated at 50°C for 10 min. DNS solution (3.0 mL) and 1.0 mL of enzyme dilution were added and boiled for 5 min. The solution was cooled in an ice water bath. Samples and the blanks were treated in the same boiling water bath. The absorbance of the enzyme sample and of the blank was measured at 540 nm against water (UV-Visible Spectrometer, Model 8453, Hewlett

Packard, Germany).

Standard curve. A stock glucose solution was prepared by dissolving 1.0 g of anhydrous glucose in deionised water to 10 mL in a volumetric flask. From this stock solution, five dilutions were prepared to yield 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL. To 1.0 mL of each glucose dilution, 1.0 mL 0.05 M sodium acetate and 3.0 mL DNS solution were added. After mixing, the enzyme sample tubes were boiled for 5 min. To prepare the reagent blank 1.0 mL deionised water was added instead of glucose solution, followed by 1.0 mL of 0.05M sodium acetate and 3.0 mL DNS solution. After mixing, samples were placed in a boiling water bath for 5 min. The absorbance of all dilutions and blanks were read at 540 nm against water. Absorbance versus milligrams of glucose was plotted and used to calculate an inverse of the slope (1/slope) and an intersection of an X - axis (C_0).

2.11.5 Extracellular xylanase assay

Xylanase activity (pentosanase/hemicellulase) was determined by the modified procedure of Bailey and Poutanen (1989). The assay is based on a 5 min hydrolysis of xylan substrate at pH 5.3 and 50°C. One xylanase unit is the amount of enzyme which liberates 1 µmol of xylose per minute under the conditions of the assay.

Reagents

Na-Citrate buffer (0.05M and pH 5.3)

Na-Citrate ($C_6H_5Na_3O_7$) (6.45 g) was dissolved in 100 mL deionised water.

Xylan-Substrate (pH 5.3)

Xylan (Sigma X-0502, St Louis, USA) (1.0 g) was warmed and made up to 100 mL with buffer. This solution was stable for 24 h when refrigerated.

Xylose Stock Solution (0.01M)

Xylose (D-xylose, Sigma X-3877, St Louis, USA) (15 g) was dissolved in 100 mL of buffer and made fresh daily.

DNS-Reagent (Dinitrosalicylic acid)

DNS (10 g) was suspended in 400 mL of deionised water and gradually added, with mixing, to 150 mL of sodium hydroxide solution (16 g NaOH per 150 mL of water). The suspension was heated in a water bath to 50°C until clear (50°C not to be exceeded). Potassium-sodium tartrate (300 g) was gradually added to the solution, with continuous mixing, and the solution was diluted to 100 mL with deionised water and stored in a dark bottle at room temperature (stable for six months).

Xylanase Test Procedure - Enzyme Preparation/Incubation

Xylan substrate (1.8 mL) was equilibrated at 50°C for 5 min. Two tubes were allowed for each sample, one for each enzyme blank and one for the substrate blank. At time zero, 0.2 mL of an appropriate enzyme dilution in citrate buffer was rapidly pipetted into the equilibrated substrate and vortexed. The tube was incubated for 5 min. After 5 min, 3.0 mL of DNS solution was added to each tube, the tubes were vortexed and removed from the water bath. Enzyme dilution (0.2 mL) was added to the enzyme blank and 0.2 mL of buffer was added to the substrate blank.

Tubes were boiled for 5 min followed by cooling for 5 min in a 21°C water bath. The absorbance was measured for each sample and blank against the substrate blank.

Standard Curve

The xylose stock solution was diluted in buffer as follows:

Undiluted (10 µmol/mL) and dilutions of 1:2, 1:4, and 1:5.

The standard curve solutions were treated the same way as the substrate blank (1.8 mL substrate plus 0.2 mL each standard), and 3.0 mL of DNS solution was added to each tube, vortexed and the tubes removed from the water bath, boiled for 5 min and then cooled for 5 min in a 21°C water bath. The absorbance was read against the substrate blank and the standard curve absorbance vs. concentration was plotted.

A standard curve was constructed for every series of assays to minimise variation due to different batches of DNS, boiling conditions, substrate solution, automatic pipette calibration and the time of assay.

Calculations

The corresponding concentration to ΔA from the standard curve ($=\Delta C$) was taken.

Activity $= \Delta A$ x dilution factor

5; μmol/mL per minute

2.11.6 Alpha amylase assay

The alpha amylase activity was determined using the Food Chemicals Codex Method (1996). Fungal alpha amylase hydrolyses the α1-4 glucosidic linkages of dextrin to yield maltose and smaller dextrins. The hydrolysed products react with an iodine solution and the colour produced is compared to a standard colour solution. As starch is broken down, the colour changes from blue to red to brown. This assay is based on the time required to obtain a standard degree of hydrolysis of a starch solution at 30°C ± 0.1 °C.

Acetate buffer (2M) was prepared by dissolving 164 g of anhydrous sodium acetate in 500 mL of distilled water. Glacial acetic acid was added (120 mL) and the pH adjusted to 4.8 with glacial acetic acid. The final volume was adjusted to one litre with distilled water.

Buffered starch solution was prepared using potato soluble starch (Sigma #2630) and 2 g was dispersed into 20 mL of distilled water. This solution was slowly poured into 60 mL of boiling water and boiled for 1-2 min with constant stirring. The mixture was transferred to a 100 mL volumetric flask, 5 mL of acetate buffer pH 4.8 was added and the volume made up to 100 mL with distilled water. This solution was prepared daily.

The enzyme dilution solution was prepared in a one litre volumetric flask by adding 0.585 g sodium chloride and 2.22 g calcium chloride to 800 mL distilled water. Acetate buffer (20 mL of 2M) was added and the pH was adjusted to 4.8 with 1M NaOH followed by filling the flask with distilled water to one litre.

Stock iodine solution was prepared by dissolving 1.1 g iodine and 2.2 g potassium iodide in 25 mL distilled water and filling a 50 mL volumetric flask to volume with distilled water. The solution was stored in the dark and a fresh solution was prepared monthly.

Working iodine solution was prepared fresh daily by dissolving 10 g of potassium iodide in 200 mL distilled water, adding 1.0 mL of stock iodine solution and bringing the solution to a 250 mL volume with distilled water.

Assay

A) Enzyme samples were diluted in enzyme dilution solution to give an end point between 10 to 20 min.

B) For each sample to be analysed, 5 mL of buffered starch solution was placed into a 20 mm x 150 mm test tube and allowed to equilibrate in a 30°C water bath for 5-10 min.

C) For each enzyme sample to be analysed, 5 mL of working iodine solution was dispensed into tubes and the tubes were held in a 30°C water bath to warm them to

30°C.

D) The spectrophotometer (Spectronic 20 Thermo Electron Corporation, Waltham, Massachusetts) was zeroed using distilled water and the absorbance of the standard colour solution at 617 nm was recorded.

F) The enzyme solution (2.5 mL) was transferred into the starch flask and mixed and incubated further at 30°C.

G) After 10 min reaction, and at definite time intervals thereafter, 1 mL of reaction mixture was placed into the 5 mL working iodine solution, mixed, and the optical density of a representative sample recorded.

H) As the optical density of the reaction mixture approached that of the colour standard, the O.D. of the reaction (as above) was measured every 30 sec until the O.D. matched the colour standard or a regression curve was made that included the O.D. of the colour standard.

One α-amylase dextrinising unit (FAU) is defined as the quantity of α-amylase that will dextrinise soluble starch at the rate of 1 g/h at 30°C and pH 4.8.

The α-amylase dextrinising units (FAU) in the sample were calculated as follows:

$$FAU/g = \frac{40 \times F}{T}$$

Where:

40 = is a constant derived from the 100 mg of starch (5 mL of a 2% solution) and the

2.5-mL aliquot of the sample preparation used (100/2.5).

T = Time of reaction in min.

F = Dilution factor for the enzyme (1000).

2.11.7 *Free Amino Nitrogen (FAN)*

The FAN was determined by the ninhydrin method 945.30 of the Association of Official Analytical Chemists (AOAC, 2002). The FAN test uses a colour reaction to detect the presence of amino acids. Ninhydrin is the reagent that reacts with free amino acids to produce a violet compound. The concentrations of FAN were analysed using a Spectronic 20 spectrophotometer (Thermo Electron Corporation, Waltham, Massachusetts).

2.11.8 *Moisture analyses*

Koji samples (2 g aliquots) were analysed for moisture content using 10.1 cm B.V.I.D aluminium drying pans (Ohaus, New Jersey) and a HG53 Moisture Analyser (Mettler-Toledo, GmbH). The samples were heated to 105°C and held at this temperature until a constant weight was achieved for a repeatability of 0.1%.

Moisture Calculation -Wet-Basis: The wet-basis moisture content was expressed as the ratio of moisture mass to the total mass of the substance. The ratio was represented by the following equation:

$$M_{wb} = \frac{m_{H_2O}}{m_{H_2O} + m_{dm}}$$

where

M_{wb} = wet-basis moisture content,

m_{H2O} = mass of moisture in kg, and

m_{dm} = mass of dry matter in kg.

Moisture Calculation - Dry-Basis

The dry-basis moisture content was expressed as the ratio of the moisture mass present in the material to the mass of the dry matter and was represented by the equation:

$$M_{db} = \frac{m_{H_2O}}{m_{dm}}$$

where

M_{db} = dry-basis moisture content,

m_{H2O} = mass of moisture in kg, and

m_{dm} = mass of dry matter in kg.

2.11.9 Water activity

Water activity was determined using a Pawkit hand held water activity meter (Decagon Devices, Pullman, Washington) with a resolution of ±0.01 a_w.

Samples were analysed in triplicate and a standard tested after every ten samples.

2.11.10 Sieve analysis

Sieve analysis was used to examine wheat bran particle size. Wheat bran batches, received in Serdán for production and experimental purposes, were assayed for particle size distribution on a routine basis using the following procedure. The sizes of the sieves used were as follows: Number 14 -1400 µm, Number 18 – 1000 µm, Number 25-710 µm, Number 35-500 µm, Number 45 -355 µm, Number 60- 250 µm, Number 80 - 180 µm, and Number 100 –150µm.Bran (100 g) was added to sieve # 14 and Sieves # 14, 18, 25 and 35 were stacked together and the sieves were shaken for 10 min. Sample coming through sieve # 35 was added to sieve # 45. Sieves # 45 to 100 were stacked together. The sieves were shaken for 10 min. Sample left in each sieve was collected and weighed.

2.11.11 pH measurement

To measure the pH of the koji, 5 g of sample was transferred to a flask and 100 mL of triple distilled water was added. The flask was shaken well to mix and after 15 min the pH was measured using a pH meter (Conductronic model 120, Puebla, Mexico).

2.12 Statistical Analysis

Statistical analyses were carried out using Microsoft Excel and the statistical package Minitab (Minitab, State College, Pennsylvania, USA).

Statistical analyses on plant scale experiments are a challenge since controlling variables in a plant is extremely difficult compared to controlling variables in laboratory flask experiments. However, results from koji flask experiments, where variables can be controlled more easily, often cannot be scaled up and reproduced in the plant environment. Differences in the microenvironment in a flask in an incubator, and the microenvironment of a tray in an incubation chamber with water cooling, are significant. A number of statistical techniques are applicable to both the plant scale trials and the koji flask trials, but the plant scale trials required the use of very specific experiments to determine what that noise level in the data is and what can be done to reduce that noise. Statistical designs described below were used to look at the following factors: tray to tray variability, within tray variability and assay to assay variability. This was to ensure that when full scale plant trials were conducted that it would be known beforehand with the chamber experiments, what the current capability of the measurement process (sampling and analysis) was. What was found during these experiments was that to overcome the noise in the system, very large numbers of samples would have to be processed. Since the processing means the destruction of the entire sample, this made following a specific fermentation over a time period complicated. It was necessary in each process to know how large a difference in signal was needed, and with what number of samples, one could be to be 95% certain that one

was detecting a true change in performance.

Assay repeatability was measured by the standard deviation of repeat assays on the same cone and quarter sample from the trays. Experimentation indicated that assay variability existed, but it was not clear if this was related to the process or to the measurement. Moisture was identified early as a possible factor in variation in the test results and experiments aimed at standardising the moisture in the samples, or compensating for it mathematically, were explored.

Nested design ANOVA (or hierarchical design) was used for experiments in which there was an interest in one set of treatments and the experimental units were measured more than once or are sub-sampled (Dowdy and Wearden, 1991). For example, if three types of bran were being tested for their effect on phytase activity, then four chambers were assigned at random (a total of twelve fermentations) to each bran, the intent being to obtain two analyses of phytase for each condition (24 determinations).

Nested design was used for some of the experimental work to address sampling strategy and to determine what had the largest impact on estimating the confidence interval (CI) of the mean.

A measurement system can differentiate between trays (process) but taking numerous measurements on one tray will not help with the estimation of the process over the entire chamber. Sampling intervals should also reflect what a customer might see in terms of how many trays make up a batch in terms of quality control standards.

Experiments were conducted to estimate the average performance by each individual tray filling batch. Analysis of the nested or hierarchical design provided estimates for the three components of variance involved.

The Latin square design is used when one wants to control the variation in an experiment that is related to rows and columns in an area. A procedure for the use of a 5x5 Latin Square Design was implemented which was ideal for the plant chamber experiments and the following protocol was followed.

Chamber locations were assigned by randomising the five columns. Three trials were selected by randomly selecting three of the total five rows. Treatments were assigned by random draw (i.e. names of treatments from a hat). Each trial (row) by itself represented a randomised block design.

Randomisation of all choices was important as it prevented possible bias, both real and perceived. The five trials gave all of the possible zone effects and each trial per week was its own randomised block and could be analysed as soon as it was complete.

In terms of procedure, the chambers were always loaded from left to right, twelve locations were sampled and 24 trays taken for analysis. The cone and quarter procedure was carried out three times on each tray and for the resultant samples the enzyme assay dilution was carried out in triplicate.

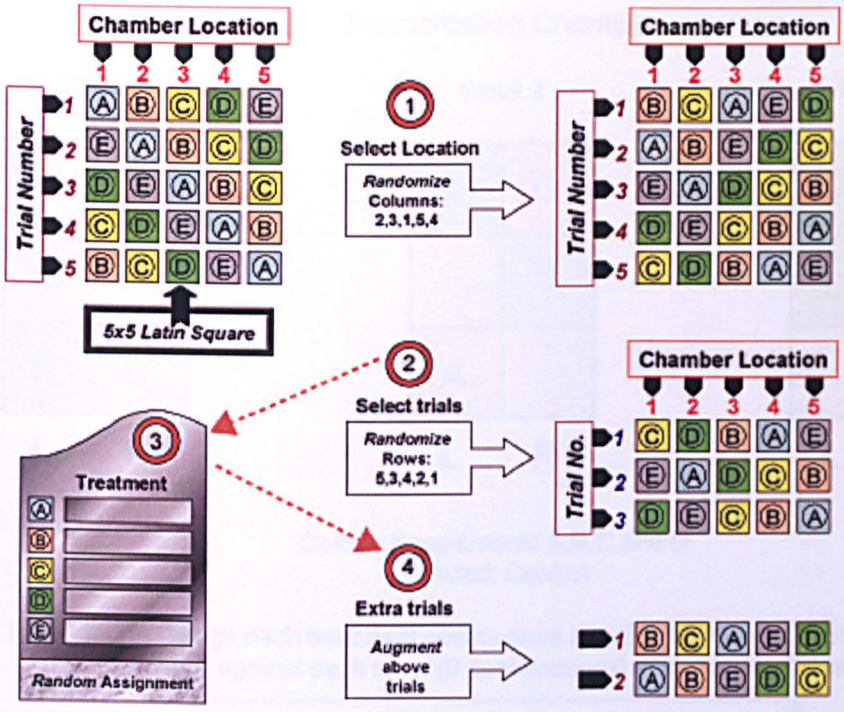


Figure 2.39 Sample 5x5 Latin square design to examine the effect of four treatment variables against a control treatment in a production chamber.

The experimental design technique of blocking, addresses the experimental error (inherent variability) encountered when replicating a trial under (supposedly) the same conditions). It is the most cost effective way of reducing the number of trials (i.e. increasing the signal to noise ratio). Blocking can be used with things such as fermentation chamber location (left/right, front, centre and back), inoculation order, day-to-day, week-to-week, etc. (Fig. 2.39).

Sampling experiments were used to address within-tray and tray-to-tray variability and regression analysis using dummy variables was a key tool. Figure 2.40 illustrates the technique of blocking of chamber location effect. The technique of randomisation is used to protect against possible bias in the treatment effects.

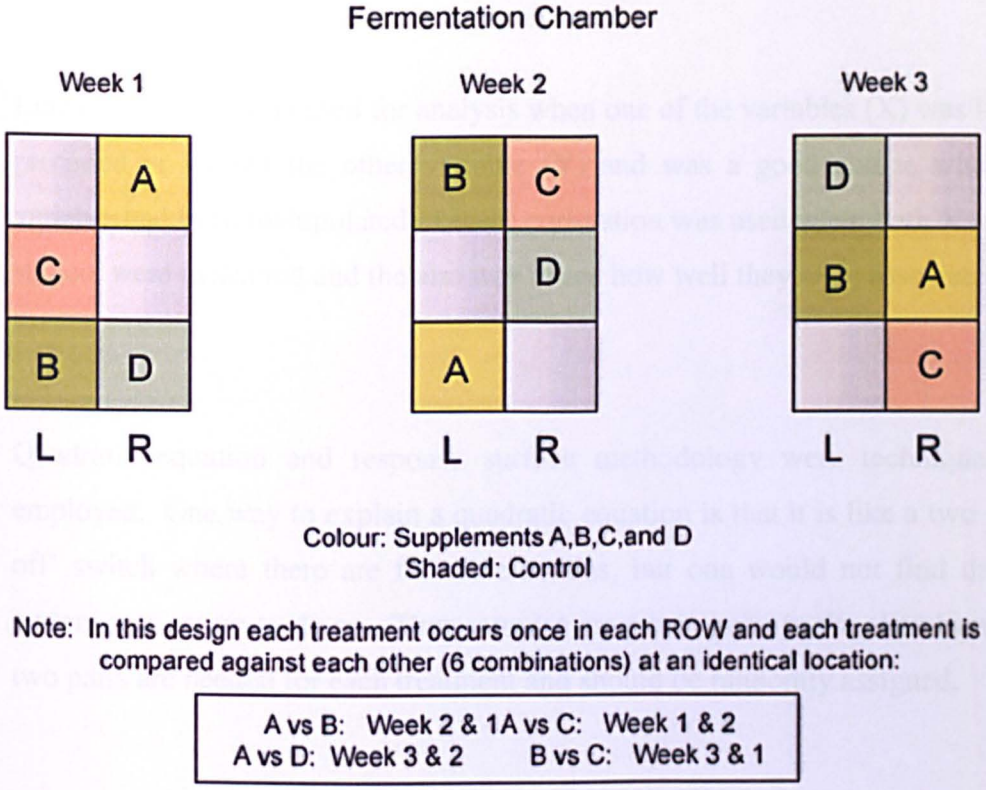


Figure 2.40 Example of technique of blocking of chamber location effect.

Response measurement concerns enzyme production. The goal is to determine what change in performance measure will be of practical significance in the plant. This is the magnitude of the signal one would look for and it is key that at the outset of the experiment one needs to be assured that the experimental design is sensitive enough to detect the practical difference specified.

Treatment effects were based on tray-to-tray differences after blocking out chamber location effects. Obtaining a representative sample within a tray was also a problem and it takes two samples to define representative sampling error. In addition, it requires two measurements on each sample to get an estimate of measurement error.

Test method repeatability was an issue and experiments were conducted to ensure that there were no issues with repeatability.

Linear regression was used for analysis when one of the variables (X) was likely to have preceded or caused the other variable (Y) and was a good choice whenever the X variable had been manipulated. Linear correlation was used when both X and Y in each subject were measured and the aim was to see how well they were associated.

Quadratic equation and response surface methodology were techniques that were employed. One way to explain a quadratic equation is that it is like a two way 'on and off' switch where there are four interactions, but one would not find them unless a system was set up to do so. Thus samples are put in pairs in the chambers and at least two pairs are needed for each treatment and should be randomly assigned.

Historically in the plant experimental data there had been too much tray to tray variability, and often the only definitive conclusion that could be drawn was that this variability existed.

Homogeneity of the samples is another concern. True homogeneity is present when one can take a sample that is 1000 g, 100 g and 1 g and the variance between the large and small sample is the same.

A central composite design using the software package, Minitab, is another way to look at the data. With contour lines it is easier to see what is happening. It allows the use of only four flasks and these could give the same information as seven trays (depending on the experimental parameter), and the experiment is less costly and requires less work to obtain the data. When the data is examined in terms of the residuals, the residuals look at the projection and how the data stands relative to the projection. In other words, how good is the model? With residuals one can look for lurking variables (unknown variables).

Contour plots let one know in which direction to move to reach the optimum. When the r-sq number is greater than 90%, then there is data that is statistically significant. With the P values, anything less than 0.05 is statistically significant.

Face - centred design Figure 2.41 requires three levels for each of two factors (i.e, the controlled variables) and is run in two stages. Blue Points (corners) with two replicates at the centre and yellow points with two replicates at the centre. This technique is useful for experiments such as those where the concentration of metal ions and their interaction require investigation.

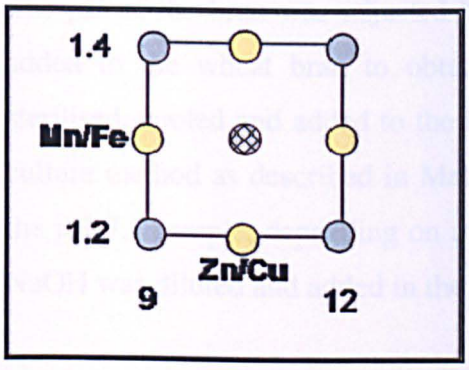


Figure 2.41 Face centred design example for interactions between trace metals.

The field of statistics is about decision making based on a group of numbers. What are the trends? What predictions can be made? No single technique is sufficient for optimisation experiments that span such a wide number of factors such as a plant process, but the use of the statistical tools described above allowed for confidence in the statistical analysis of the data.

2.13 Koji Flask and Chamber Experimental Procedures

2.13.1 Koji flask experiments with variables of zinc content, pH, moisture and temperature

Zinc addition

Zinc acetate $(CH_3COO)_2$ $Zn{\cdot}2H_2O$ (Fermont, Mexico), was added to distilled water, the pH was adjusted, the solution sterilised and added to the 10 g of sterile wheat bran along with the standard koji inoculation medium in a 200 mL flask.

Adjustment of pH koji flasks

The pH of the bran was adjusted by the addition of a dilute HCl solution. This was added to the wheat bran to obtain the desired starting pH. The wheat bran was sterilised, cooled and added to the flasks and inoculated using the standard inoculation culture method as described in Methods Section 2.6. For the pH 6.2 - 6.4 sample and the pH 7.5 sample, depending on the starting pH, either no adjustment was required or NaOH was diluted and added in the same manner as the HCl.

Moisture adjustment

Moisture in the koji flasks was adjusted to 46%, 50% and 54% by adding additional sterile water to the koji flask starter medium.

Incubation temperature

Flasks were incubated at 30°C ± 1°C for 96 h and 120 h using the laboratory incubation room. Koji flasks were removed after 96 h and 120 h and assayed for final pH, moisture and phytase activity. The experiment involved the analysis of 108 koji flasks.

2.13.2 Manganese, zinc and copper supplementation in koji flasks

A set of how 72 koji flasks containing manganese, zinc acetate and copper at different levels was inoculated with *A. niger* liquid inoculum and incubated at 30°C and 35°C. The fermentation products were analysed after four days to examine the effect of the mineral combinations on the production of phytase enzyme. All flasks were prepared and inoculated following the standard procedure in Methods Section 2.8.1.

Mineral supplementation consisted of zinc acetate $(CH_3COO)_2$ $Zn{\cdot}2H_2O$ (Fermont, Mexico), 50 and 500 ppm, cupric sulphate $CuSO_4{\bullet}5H_2O$ (OMNICHEM, Mexico) 30 and 300 ppm, and manganese acetate $(CH_3COO)_2$ $Mn{\cdot}4H_2O$ (Fermont, Mexico) 20 and 200 ppm.

The wheat bran used for this experiment had the following particle size distribution. It contained 25% large particles (mesh 14) and 70% medium range particles (mesh 35 and 60) and 5% of sieved wheat bran through mesh 100.

Treatments were prepared by adding the mineral supplements to 500 g of wheat bran and distributing the mixture into nine koji flasks, each containing 10 g of the bran mixture, and inoculating the flasks with the same starter culture grown in liquid medium as described in Methods Section 2.6.2.

Eight treatments were examined, each with nine flasks per treatment. One flask was used to analyse for starting pH and moisture. The flasks were incubated as described in Methods Section 2.8.1. After 96 h the koji was removed from the remaining eight flasks of each treatment, pooled and dried for 90 min at 42°C in an oven dryer to ~10% moisture. Samples were cone and quartered and three samples of 5 gram removed and rehydrated with acetate buffer for 60 min at 250 RPM and analysed for phytase activity. Another sample was removed for pH analysis and another one removed for moisture

analysis after drying.

2.13.3 Multivariable experiments in chambers

Koji in trays were prepared to test four variables (initial moisture level, initial pH, zinc level and time) following the standard procedures outlined in Methods Section 2.8.1. Zinc acetate addition for trial 1 was 0 ppm, 3.6 ppm and 7.2 ppm and for trial 2 the addition was 0 ppm, 69 ppm and 138 ppm. Starting pH not adjusted and initial moistures were adjusted to 46, 50 and 54%. Incubation temperature was 35°C. Koji trays were removed after 96 h and 120 h and assayed for final pH, moisture and phytase activity. Each experiment involved the analysis of 108 trays. Analyses for phytase were conducted in duplicate.

2.13.4 Time course and location experiments in chambers

Samples were selected for assay from a full fermentation chamber (Fig. 2.42) where the bran had been inoculated with *A. niger* and incubated for phytase production using the standard techniques described in the Methods Section 2.8.2. Six trolleys were filled from one batch of inoculated koji. The chamber was filled with six separate tray fillings. The selected tray filling and its respective six trolleys were marked and monitored daily for four days by testing 24 trays daily for water activity, pH, and moisture (single analyses). On Day 4, 24 trays were removed from the chamber, cone and quartered three times and the representative samples assayed for phytase activity. Samples were not dried and activities were expressed as wet activity. All phytase analyses were carried out in duplicate.

Since sampling is a destructive process, once the tray has been selected for sampling, it is used in its entirety. For Trolley Set E, the trolleys had to be moved after day 4 and were relocated in incubation chamber 4, where the resident trolleys were already at 48 h of fermentation (high heat production should have peaked). Sample trays for days 5, 6 and 7 from Trolley Set E were analysed for phytase, water activity, pH, and moisture.

The trolley locations in the chamber and the specific trays sampled are identified in Figure 2.43.

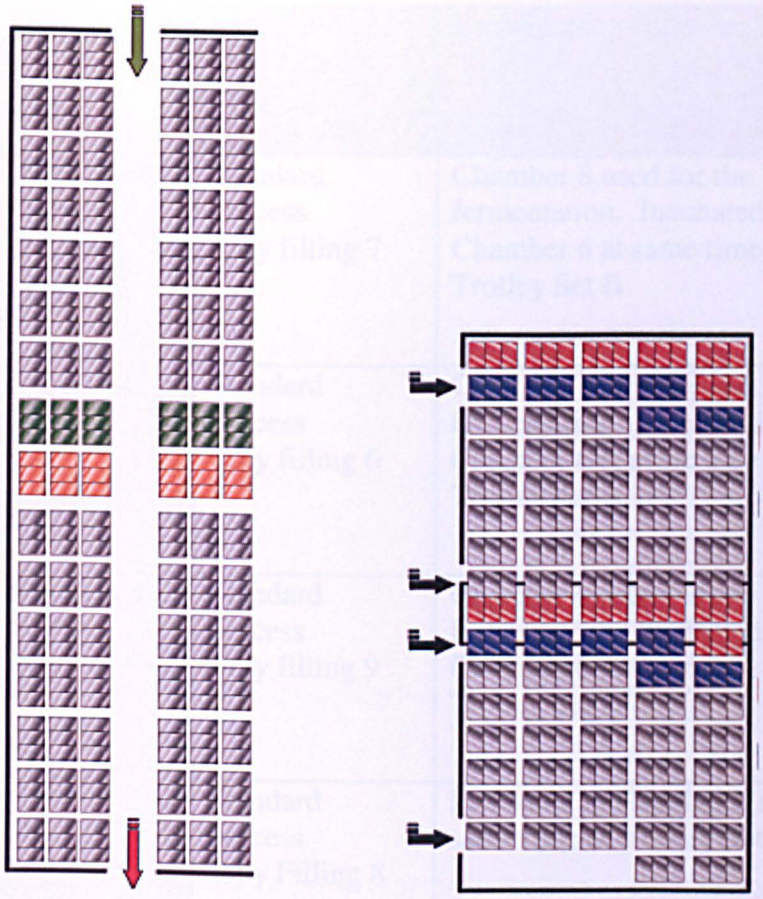


Figure 2.42 Location of trolleys sampled in the fermentation chamber.

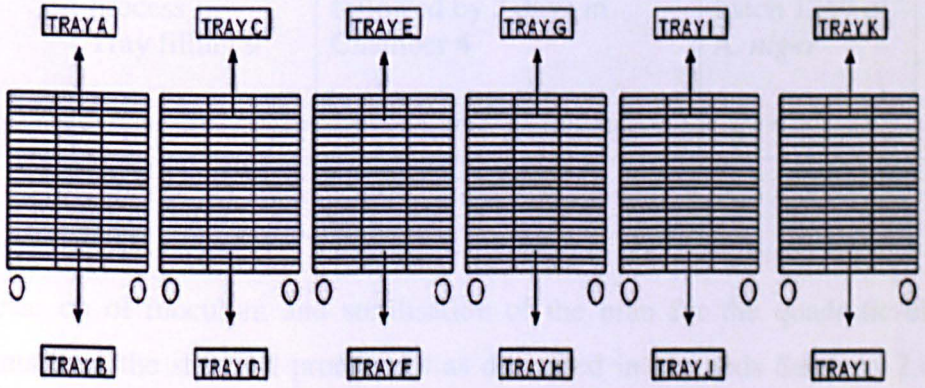


Figure 2.43 Location and labelling of trays on trolleys for sampling.

Five separate large scale trials were conducted and the differences between the trials are illustrated in Table 2.7 below.

Table 2.7 Description of trolley sets.

Tray Group	Inoculation & Fermentation Process	Fermentation Chamber	Liquid Starter Inoculum	Initial pH of the Wheat Bran
Trolley Set A	Standard process Tray filling 7	Chamber 8 used for the fermentation. Incubated in Chamber 6 at same time as Trolley Set B	Starter culture batch 1267 of *A. niger*	Starting pH: 6.1
Trolley Set B	Standard process Tray filling 6	Chamber 8 used for the fermentation. Incubated in Chamber 6 at same time as Trolley Set A	Starter culture batch 1267 of *A. niger*	Starting pH 6.1
Trolley Set C	Standard process Tray filling 9	Chamber 6 used for the fermentation. Incubated in Chamber 6 at same time as Trolley Set D	Starter culture batch 1269 of *A. niger*	Starting pH 6.5
Trolley Set D	Standard process Tray Filling 8	Incubated in chamber 6 at same time as Trolley Set C	Starter culture batch 1269 of *A. niger*	Starting pH 6.5 - pH raised by addition of NaOH
Trolley Set E	Standard process Tray filling 9	First 4 days in Chamber 6 followed by 3 days in Chamber 4	Starter culture batch 1269 of *A. niger*	Starting pH 6.1

Liquid scale up of inoculum and sterilisation of the bran for the quadratic chamber experiments used the standard procedures as described in Methods Sections 2.4.2 and 2.4.3.

Chapter 3: Results

3.1 Mass and Energy Balance

Mass balances (or material balance) and energy balances are important ways for a system to be measured for efficiency and are critical for process optimisation. By taking into account the total balance of the system, it is easier to see where these efficiencies can be realised.

Mass balance is rooted in the law of conservation of mass/matter or the Lomonosov-Lavoisier law. This states that mass in a closed system of substances will remain constant. It cannot be created nor destroyed but may change forms. Therefore, the mass of the reactants in a process must equal the mass of the products. Equally, the theoretical basis for an energy balance comes from the first law of thermodynamics, which similarly states that energy cannot be created or destroyed, only changed in form.

Energy balances are very similar to mass balances but there are some important differences to point out. A system that may be closed in terms of a mass balance could be open as an energy balance. Also, there can be several mass balances in a system, but only one total energy balance. Energy cannot be produced in a system, just converted. Therefore, when written as inputs and outputs, energy balances can be written as Input = Output + Accumulation. However, in the case when heat balance is the focus, an energy balance can also be written Input + Generation = Output + Accumulation.

The approach of using mass and energy balances in the scale up operation of bioreactors in industrial applications of SSF has generated important advances. According to this approach, the most important parameters that need due consideration during scale up of a SSF bioreactor include agitation (turbulence), aeration and oxygen transfer, temperature of the substrate bed, moisture content in the bed and finally humidity of the

bioreactor (Raghavarao *et al.* 2003).

The difference between a SSF bioreactor system and a tray system are significant, but many of the same principals apply. A tray system must be measured based on similar aspects including aeration and flow of air, air humidity and temperature, temperature of the tray reactors, substrate bed depth, substrate moisture and water flow and temperature. Both systems operate in a pseudo steady state as minor losses of energy can be expected through convection through the walls of the chambers or the bioreactor walls itself.

A trial was carried out in 2002 at the Universidad Autonoma Metropolitana (UAM), Iztapalapa, Mexico City, Mexico as a cooperative effort between the UAM and Alltech. The emphasis was to calculate the number of joules produced by *A. niger* in the SSF system. Table 3.1 shows the results from this trial and breaks down the data into how much heat is produced per gram of product and per cubic meter. The statistical analysis came from twelve trials and generated a ±8% of error in the calculations.

Table 3.1 Results of heat generated in phytase production at laboratory scale.

Units	Total Joules	J/g	KJ/m^3
Accumulated Heat	15,700	47	17,320
Convective Heat	52	0.15	57
Evaporative Heat	132,630	393	146,100
Metabolic Heat	148,382	440.15	163,477

After these results were received, similar trials on a production scale in Alltech Serdán were set up to verify the data and compare with a production scale trial. In SSF, the metabolic heat produced during SSF is estimated indirectly by an energy balance of the

fermentation chamber. All necessary parameters are measured in order to calculate the metabolic heat development with the aim to obtain information about the metabolic heat flow over the time course of the fermentation.

A trial to estimate the metabolic heat produced during SSF was carried out in the experimental chamber at Serdán. This chamber is roughly a third the size of a normal chamber and is discussed in Methods Section 2.8.4. A laboratory set up in a controlled environment offers a more exact determination of metabolic heat development during solid fermentation. However, in order to test the efficiency of a system, plant scale trials must be carried out. The metabolic heat is estimated indirectly by an energy balance of the fermentation chamber (Fig. 3.1). By thinking of the chamber as a black box, the inputs and outputs during the process can be limited to air and water. During fermentation, water is necessary to heat the tray to the desired fermentation temperature at the start of fermentation and for cooling of the fermentation chambers during peak metabolic heat production. Air provides sufficient oxygen to the organism and also removes some heat. By monitoring the flow of water and air, in and out of the chamber, the amount of heat being generated can be estimated.

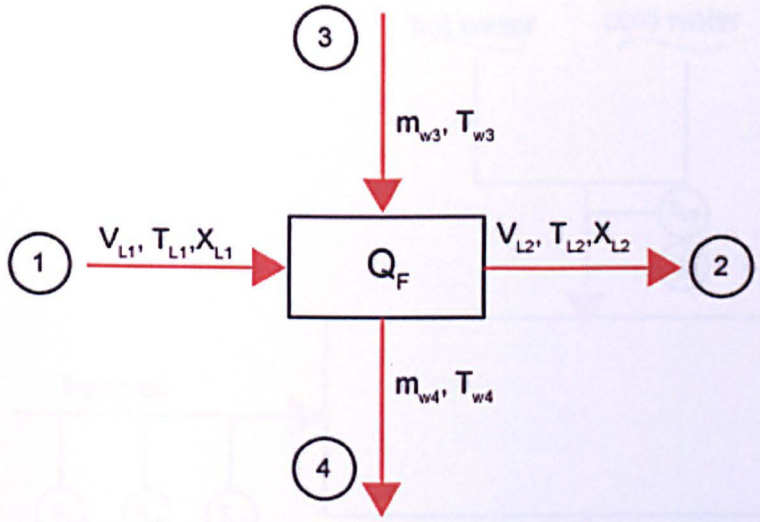


Figure 3.1 Mass and energy balance for fermentation chamber.

Air mass balance: $\dot{m}_{L1} = \dot{m}_{L2}$ (1)

Water balance: $x_1 \cdot \dot{m}_{L1} + \dot{m}_{w3} = x_2 \cdot \dot{m}_{L2} + \dot{m}_{w4}$ (2)

Energy balance: $\dot{m}_{L1}(h_{1+x})_1 + \dot{m}_{w3} h_{w3} + \dot{Q}_F = \dot{m}_{L2}(h_{1+x})_2 + \dot{m}_{w4} h_{w4}$ (3)

A trial was conducted in the experimental chamber which measures 3.7m x 14m x 3.6m with a volume of 186m^3. The space proportions are identical in set up to a normal chamber, however, it is one third the size. Also air flow into this area is more restricted than in the normal fermentations chambers. Measurements were taken during the solid state fermentation of the *A. niger* culture. Three tray fillings were loaded into the chamber on five trolleys per tray filling with 40 trays on each trolley resulting in fifteen trolleys with 600 trays in total. The total weight of koji per tray filling was 2640kg (480kg wheat bran + 400 L H_2O = 880kg x 3 = 2640kg). The fermentation was carried out under standard conditions with a temperature set point of 30°C.

In Figure 3.2 a schematic of the locations for measurement in the system are described. The water system has hot and cold water mixed to create an inlet water system.

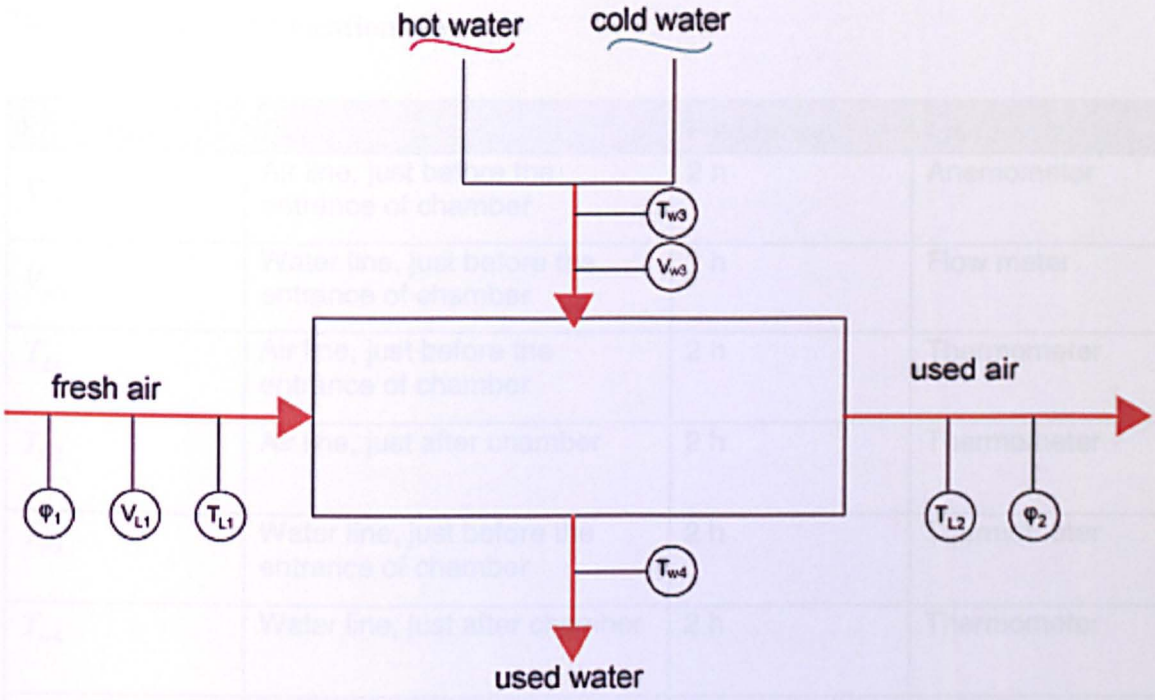


Figure 3.2 Schematic of measurement of air and water flows into a chamber.

Flow, temperature and humidity were measured throughout the fermentation in a manner described in Table 3.2 on the four separate flows in the system, air and water inlets and air and water outlets. Four different measurement tools were used. An anemometer was used for air flow, a flowmeter was used for water flow, a thermometer was used for all temperatures, and a hygrometer was used for humidity. These measurements were taken in order to calculate the actual phenomenon taking place in the fermentation.

Table 3.2 Sampling location plan.

Parameter	Place	Frequency	Instrument
$\dot{V}_{L1}$	Air line, just before the entrance of chamber	2 h	Anemometer
$\dot{V}_{m3}$	Water line, just before the entrance of chamber	2 h	Flow meter
T_{L1}	Air line, just before the entrance of chamber	2 h	Thermometer
T_{L2}	Air line, just after chamber	2 h	Thermometer
T_{w3}	Water line, just before the entrance of chamber	2 h	Thermometer
T_{w4}	Water line, just after chamber	2 h	Thermometer
φ_1	Air line, just before the entrance of chamber	2 h	Hygrometer
φ_2	Air line, just after chamber	2 h	Hygrometer

Measurements were taken throughout the process in the manner described in Table 3.2. All calculations and measurements can be found in Appendix B to this document.

Water usage throughout the fermentation is described in Figure 3.3. The bulk of the water was used between 46-70 h.

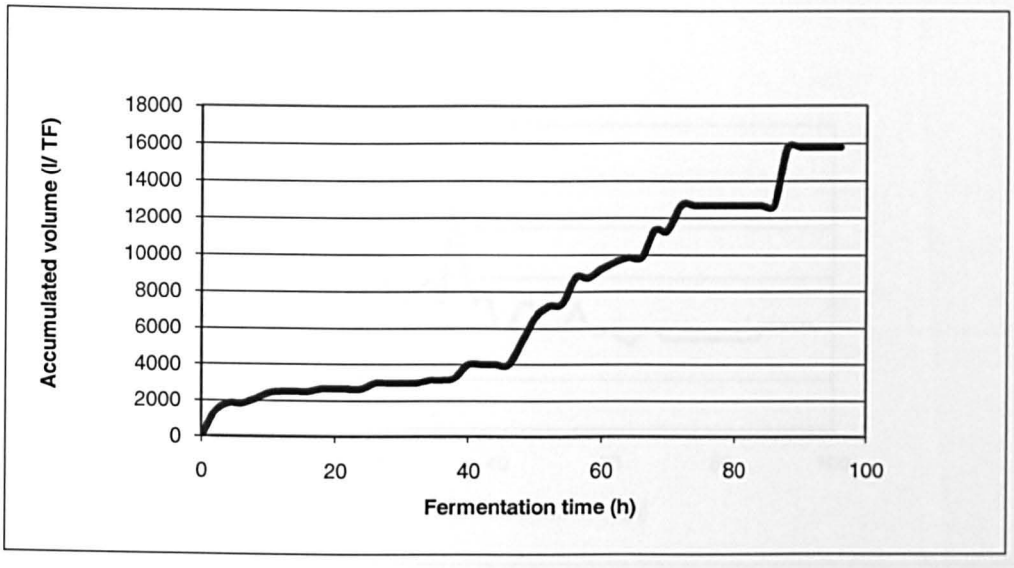


Figure 3.3 Water consumption during a 96 h fermentation of *A. niger*.

Along with this flow measurement, the water temperature must be considered. Figure 3.4 shows the water temperature throughout the fermentation. At the start of the fermentation, hot water was utilised to raise the temperature to the set point. From there, water was the primary cooling agent in the process. Due to cooling constraints in this experiment, the water temperature does not fall much lower than 20°C and therefore the water flow was relied upon as the major factor influencing cooling in this experiment.

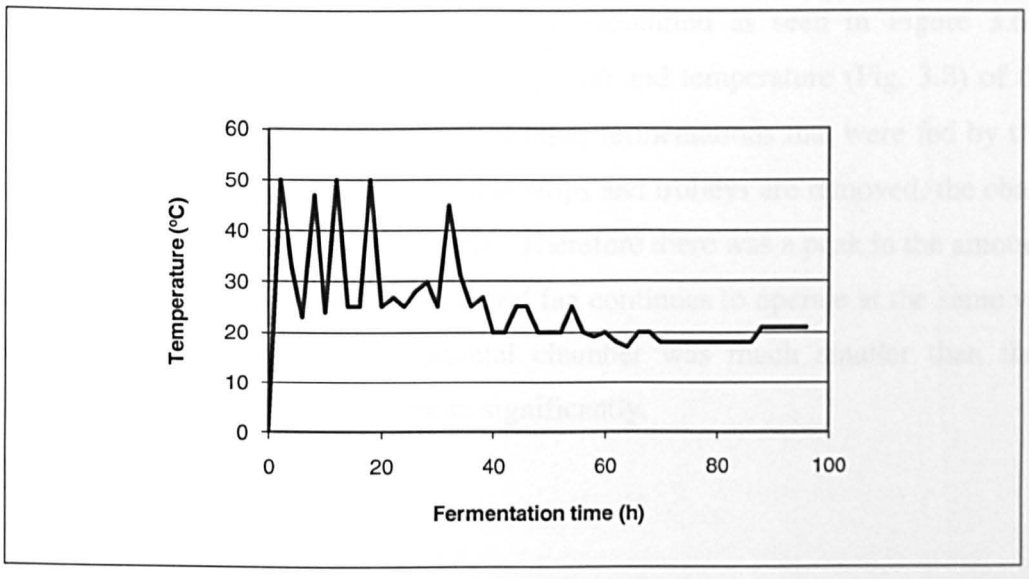


Figure 3.4 Water temperature (inlet) over a 96 h fermentation with *A. niger*.

The outlet water temperature can be looked at to reflect the efficiency of cooling. Comparing the inlet temperature with the water outlet temperature in Figure 3.5, it can be seen that water around 50 h has picked up a large amount of heat, while towards the end of the fermentation it has not significantly increased in temperature.

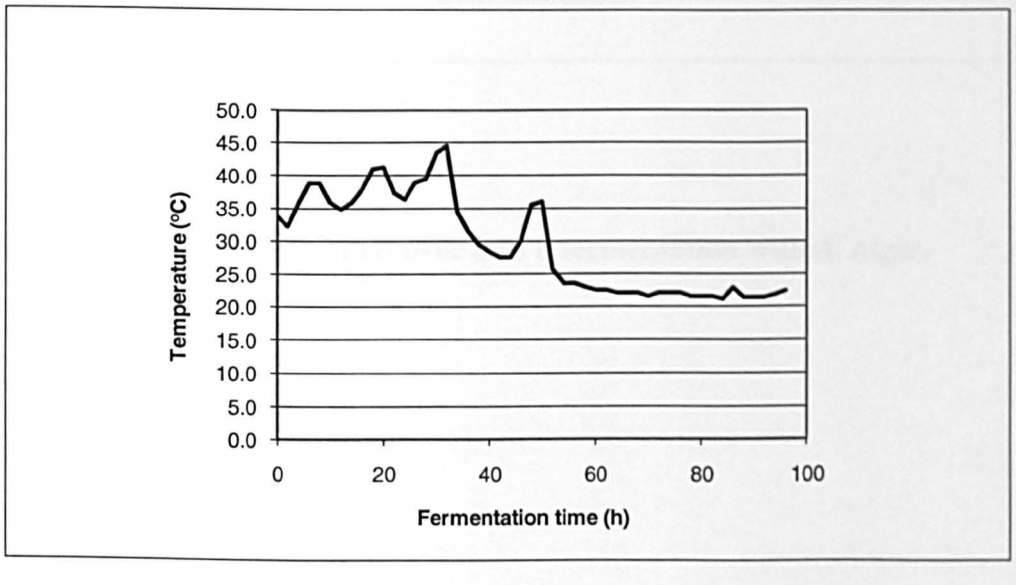


Figure 3.5 Water temperature (outlet) during a 96 h fermentation with *A. niger*.

Air velocity inlet varies throughout the fermentation as seen in Figure 3.6. This variation was related to the humidity (Fig. 3.7) and temperature (Fig. 3.8) of the inlet air. It also changed due to the status of other fermentations that were fed by the same central air system. When a fermentation stops and trolleys are removed, the chamber is cleaned and the air system is closed off. Therefore there was a peak in the amount of air available for other chambers. The central fan continues to operate at the same velocity. Due to the fact that the experimental chamber was much smaller than the other chambers, these effects are seen more significantly.

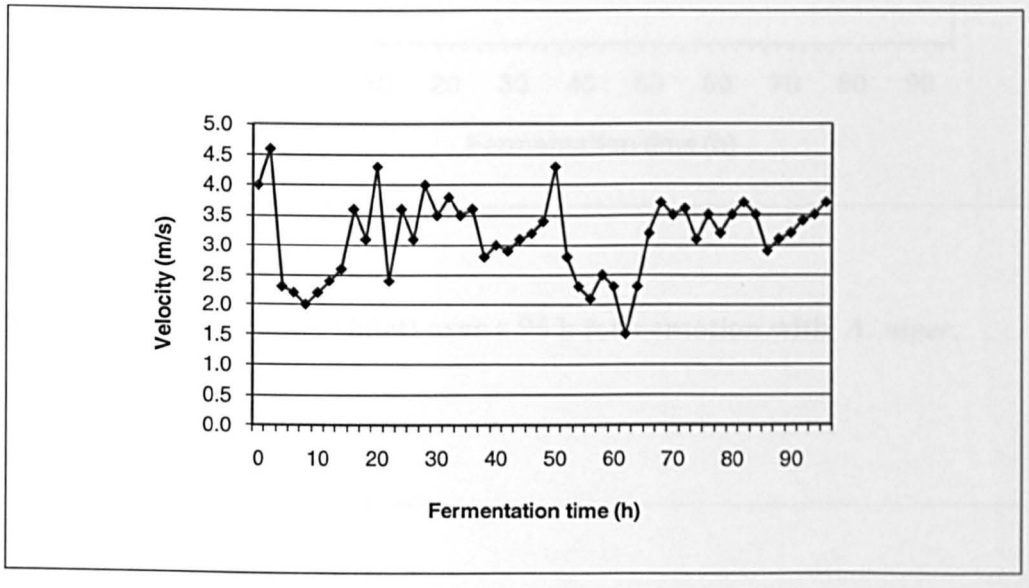


Figure 3.6 Air velocity (inlet) over a 96 h fermentation with *A. niger*.

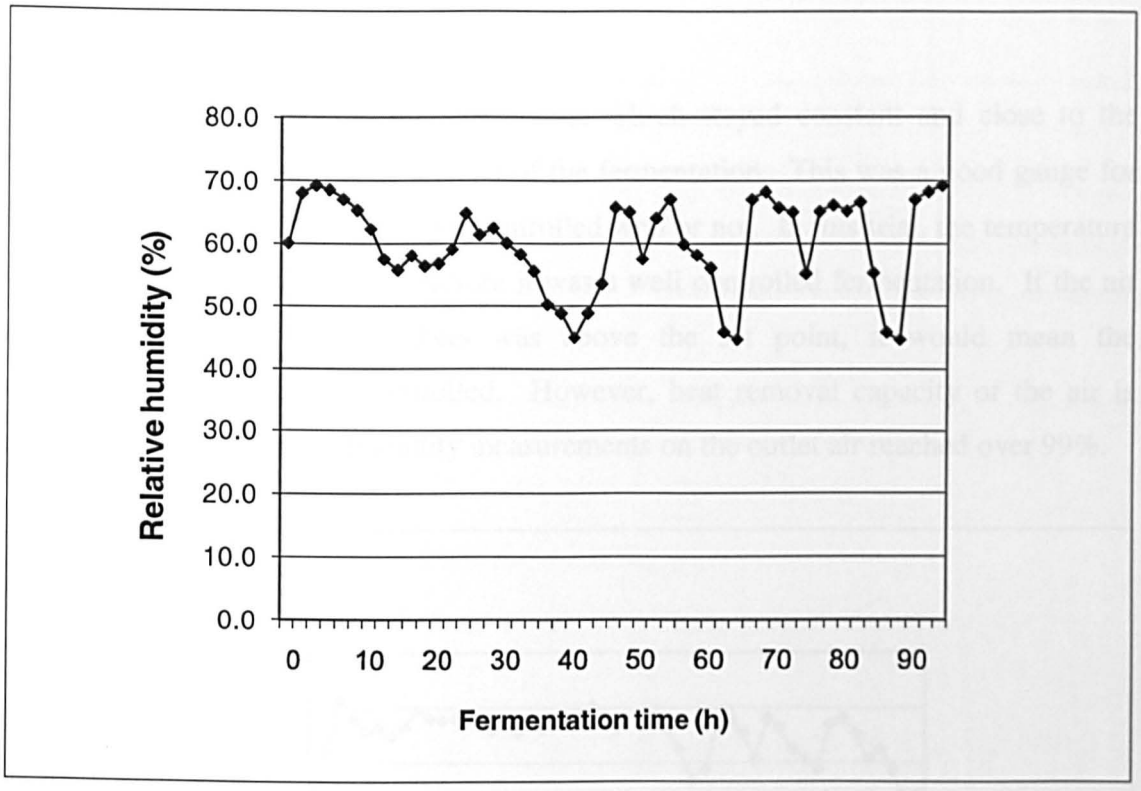


Figure 3.7 Air humidity (inlet) over a 96 h fermentation with *A. niger*.

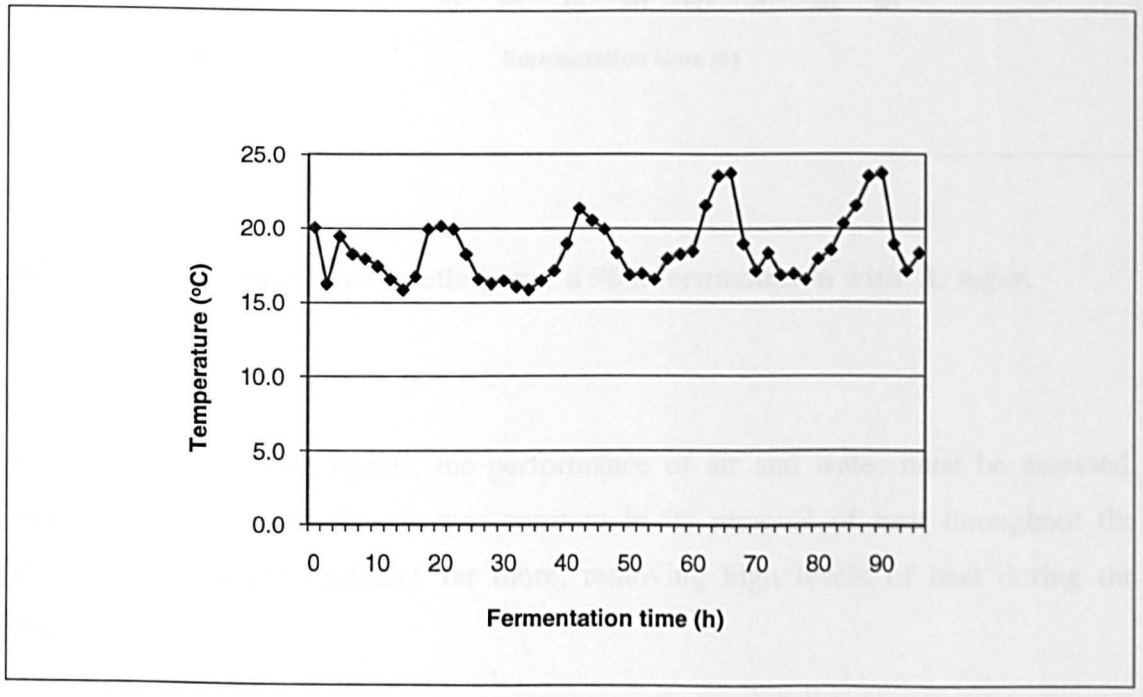


Figure 3.8 Air temperature (inlet) over a 96 h fermentation with *A. niger*.

Figure 3.9 shows the outlet air temperature which stayed constant and close to the fermentation temperature until the end of the fermentation. This was a good gauge for whether the fermentation was being controlled well or not. In this trial, the temperature was consistently under 30°C, therefore it was a well controlled fermentation. If the air temperature leaving the chambers was above the set point, it would mean the fermentation was not well controlled. However, heat removal capacity of the air is limited due to the fact that humidity measurements on the outlet air reached over 99%.

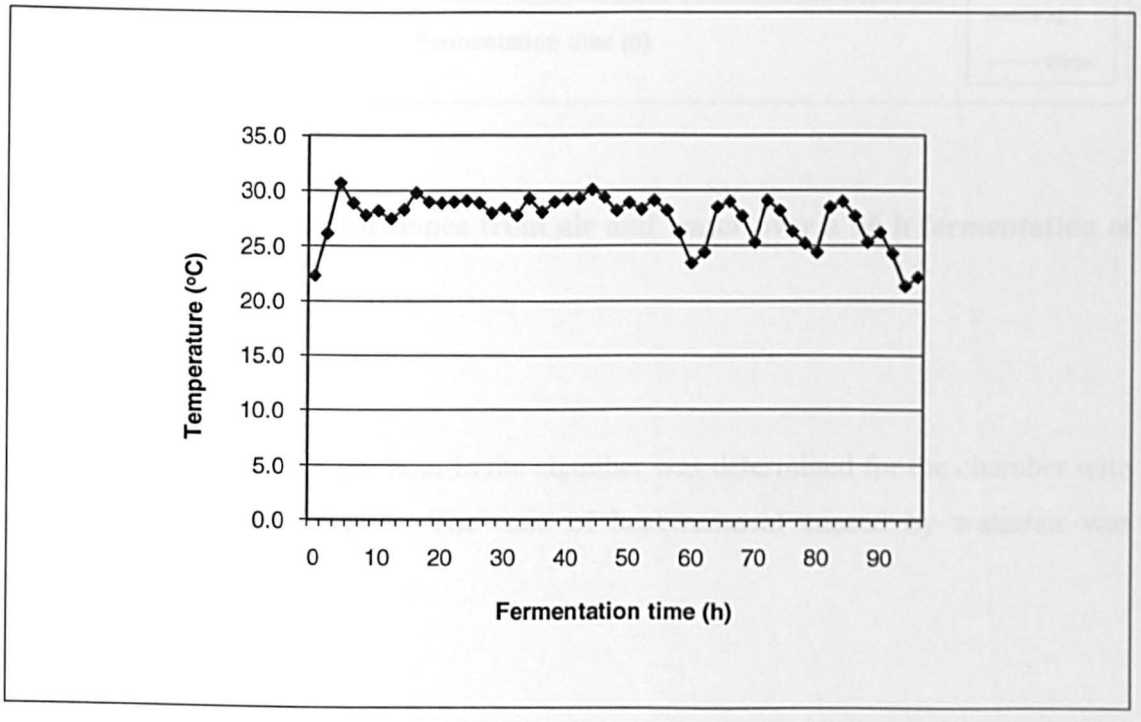


Figure 3.9 Air temperature (outlet) over a 96 h fermentation with *A. niger*.

As the primary cooling agents, the performance of air and water must be assessed. Figure 3.10 shows that the air was constant in its removal of heat throughout the fermentation. Water fluctuates far more, removing high levels of heat during the fermentation peak.

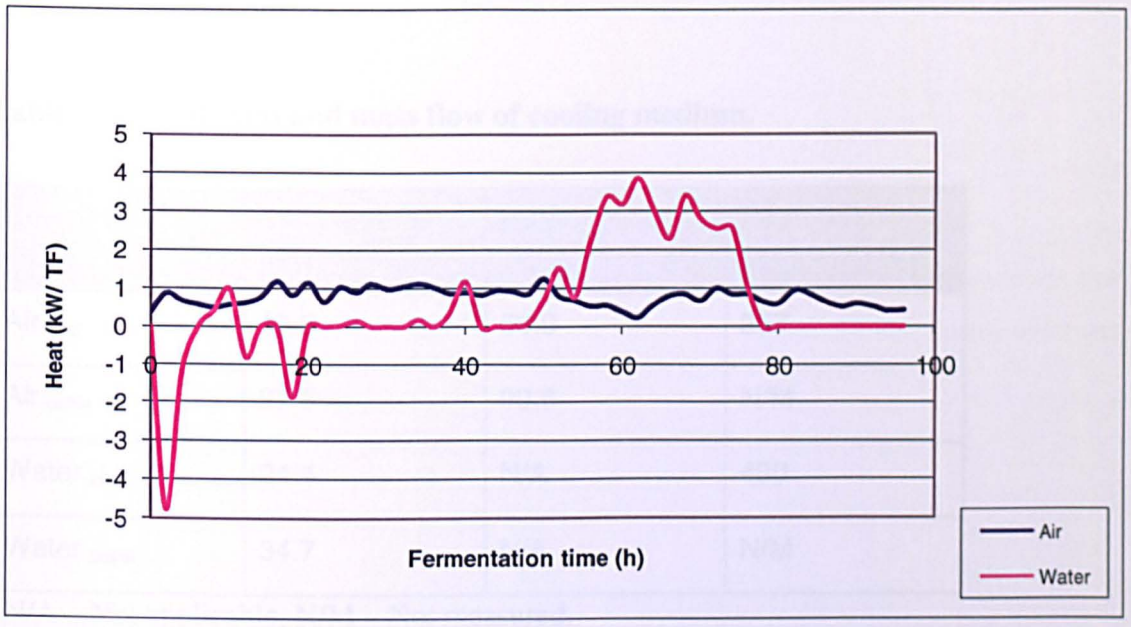


Figure 3.10 Cooling performance from air and water over a 96 h fermentation of *A. niger*.

The change of air volume per hour in the chamber was determined for the chamber with 1.38 changes per hour (cph). The ratio of heat removal caused by water/air was determined as 65% /35%.

During the heat peak of fermentation (56-70 h) the ratio of heat removal caused by water/air changed to 85% /15% as more water entered the chamber. The condition of water and air entering and leaving the chamber was measured as well as the corresponding mass flows (Table 3.3).

Table 3.3 Conditions and mass flow of cooling medium.

Description	Temperature (°C)	Humidity (%)	Mass flow [(kg/h), (m^3/h)]
Air $_{inlet}$	18.5	59.9	257
Air $_{outlet}$	27.5	99.6	N/M
Water $_{inlet}$	24.4	N/A	490
Water $_{outlet}$	34.7	N/A	N/M

N/A – Not applicable, N/M – Not measured.

The maximum metabolic heat release needs to be considered, since it is this level that must be removed in order to control the temperature of the fermentation throughout its duration. The peak heat occurred during 56-70 h of fermentation and was determined with 4.08 kW/ tray filling (TF). If air is used as the sole source of cooling the chamber, an air flow of 470 m^3/h/TF is required. This translates for the current fermentation chamber in use into approximately 2.7 cph /TF.

During fermentation an average metabolic heat release of 1.7 kW/TF was measured. This means that a total metabolic heat release of 576,000 kJ per tray filling was produced during solid fermentation with *A. niger* or 1378 kJ per kg wheat bran. The calculation used for this study is listed in Figure 3.11.

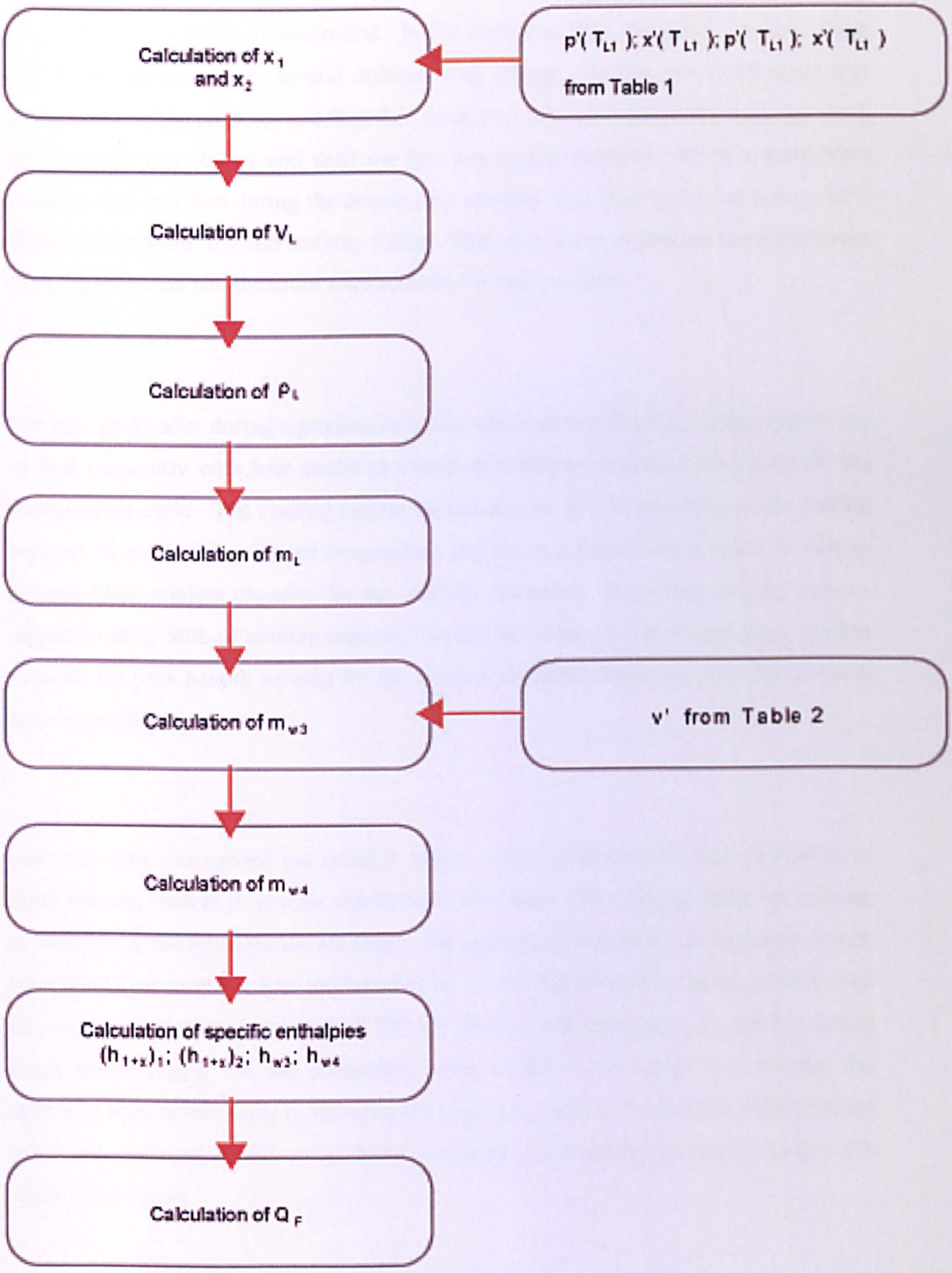


Figure 3.11 Calculation programme.

Earlier results have shown that temperature control is a critical parameter in SSF (Schutyser, 2003). It is clear that heat production needs to be calculated in order for the temperature to be properly controlled. In the present system, chambers are quite large and include product from several different tray fillings. In this case, only three tray fillings were in the chamber and therefore there was only a 3 h difference between when the first one was started and until the last was in the chamber. Since a production chamber will be filled during the course of a working day, there can be at least a 12 h difference between first and last tray filling. This could have significant impact in terms of heat production and therefore requirements for heat removal.

The heat peaks alter during a production week, which means that the cooling system has to deal constantly with heat peaks produced in different chambers depending on the fermentation cycle. The cooling system should also be able to provide enough cooling capacity to enforce the desired temperature profile on a fermentation batch as well as provide basic cooling capacity for the residual chambers. Separated cooling systems (approximately 50% of cooling capacity) should be added on top of maximum cooling capacity for peak heat to account for the residual chambers which are fermenting wheat bran in parallel.

Also this trial was carried out using *A. niger*, which produces little heat compared to other species, such as *A. oryzae*, discussed in this study. This fungal species is difficult to maintain at the fermentation set point. The maximum volume of cooling water which is available per chamber was estimated to be 2.7 l/s. Therefore the cooling systems can take out maximum heat removal of 100 kW during peak production at typical cooling water temperatures. In the production scale, chillers were added to overcome the difficulty seen in this study in reducing the water temperature for cooling. This reduced water requirements significantly, which outweighed the energy needed to reduce the water temperature.

3.2 Water and Air Supply and Heat Removal

Analysis for optimisation of water use in chambers.

The objective was to use conditioned air to cool the chamber. The use of water is restricted to keep air saturated and thus avoid the koji in the chamber from drying out. The air is conditioned with steam.

Calculation of required air volume in order to achieve a CO_2 concentration of 2% (v/v):

Assumption: 10kg of wheat bran produces 2kg CO_2 during the five days (120 h of fermentation). The chamber contains 4,800kg of wheat bran when fully loaded. Therefore, the mass flow of CO_2 = 80kg/h which gives approximately a volume flow of CO_2 at 40 m^3/h (assuming ideal steady state).

In order to reach a CO_2 concentration of 2% (v/v), 2000 m^3/h of air is required. This volume flow could not be changed in the process. Therefore, the solution was to adjust the required air temperature in order to cool chambers by conditioning the air rather than adjusting the volume flow of the air. This should be possible by conditioning air with steam for heating and spray water for cooling.

Calculation of air temperature into the chambers and mass flow of dry air:

Assumption: 1kg of wheat bran produces 100 kJ/day. Consequently, approximately 6 kW should be produced by the koji. Furthermore, if one takes the surface of the trays with a fully loaded chamber of 85 m^2 and alpha air = 10 W/m^2.K, the estimated air temperature would be 24°C. The air will heat up and the relative humidity of air decreases. The humidity of the air would be balanced by adding water into the chambers.

Calculation of water mass and water temperature for the air conditioning.

Assumption: Condition of air from outside (T= 20°C, RH= 50%). The amount of steam depends on steam temperature and water content to condition the air (T=24°C and RH= 100%).

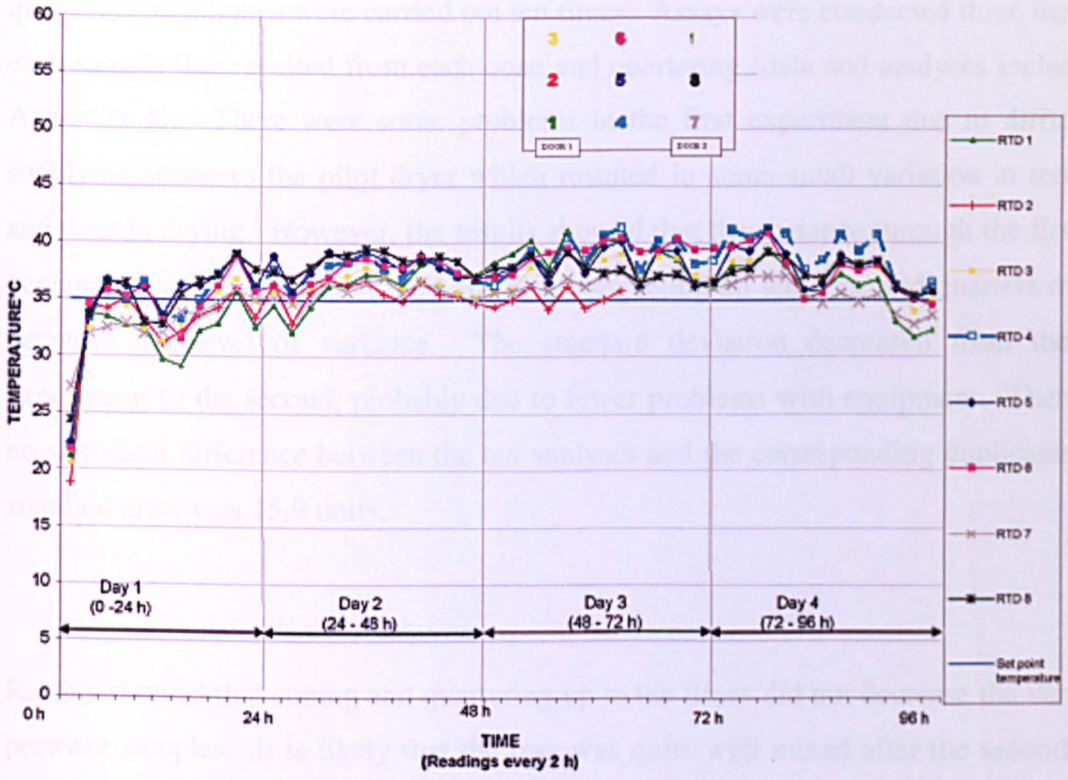


Figure 3.12 Typical temperature profile of fermentation chamber over 96 h.

Figure 3.12 illustrates a typical fermentation temperature profile. Measurements were made every 2 h by RTD's located at eight different spots in the chamber as illustrated on the diagram.

3.3 Sampling Techniques and Standardisation

In order to assure sampling and assay reproducibility, standard methods were tested.

3.3.1 Sampling reproducibility (cone and quartering)

An experiment was carried out in duplicate to test the current cone and quarter practices to determine if this was a source of variance in the analyses. The experimental procedure is described in detail in Methods Section 2.10.1. Samples were taken from four trays on the same trolley and each tray was coned and quartered. These cone and quartering techniques were carried out ten times. Assays were conducted three times on each sample that resulted from each cone and quartering (data and analyses included in Appendix E). There were some problems in the first experiment due to difficulties supplying steam to the pilot dryer which resulted in some small variation in moisture and time in drying. However, the results showed that the variance through the first five cone and quarters was very small and that completing all ten cone and quarters did not decrease the level of variance. The standard deviation decreased from the first experiment to the second, probably due to fewer problems with equipment. There was no statistical difference between the ten analyses and the corresponding duplicates. The standard error was 15.9 units.

Results showed that coning and quartering up to ten times did not decrease the variation between samples. It is likely that the tray was quite well mixed after the second cone and quartering and that the assay has a reasonable and acceptable level of variation.

The conclusion from these experiments was that the assay has some variance, the sampling does not show a significant difference and the trays have variance and operate almost like mini batches. By cone and quartering samples ten times and assaying each one in triplicate, it could be derived that the ideal number of times to cone and quarter would be three.

3.3.2 Enzyme assay reproducibility/ assay

An experiment was conducted to determine the assay capability. Eight production koji trays were sampled for phytase activity and for each koji tray there were six cone and

quarter samples and these were assayed in triplicate (for a total of 144 samples) following the procedures outline in Methods Section 2.10.1. Analysis of the data indicated that 94% of the assay results were within ±15 units and 80% of the data fell between within ± 10 units (Table 3.4).

Table 3.4 Data analysis of assay capability from the Excel data analysis tool histogram.

Bin	Frequency		
-25	0		
-20	1		
-15	9		
-10	11		
-5	23		
0	26	115	136
5	35	80%	94%
10	20		
15	12		
20	4		
25	3		
More	0		
Total	144		

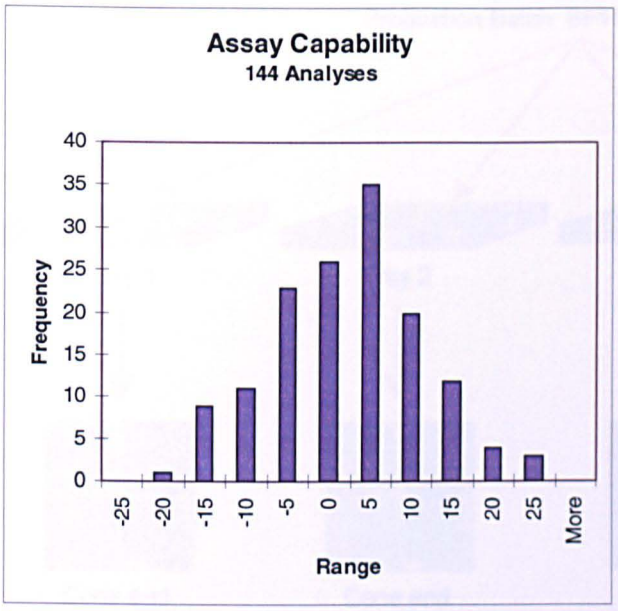


Figure 3.13 Histogram illustrating assay capability distribution.

The assay capability is also shown as a histogram in Figure 3.13. Determining the assay capability was a very important step in understanding from where the variation in data was arising.

Assay reproducibility and sample storage at 4° C and 21° C

Studies were carried out to determine how best to store samples for analysis for phytase activity, since samples were often stored and then processed as one batch to eliminate operator and reagent variables. It was of interest to determine whether there was an influence on phytase enzyme activity due to storage before analysis.

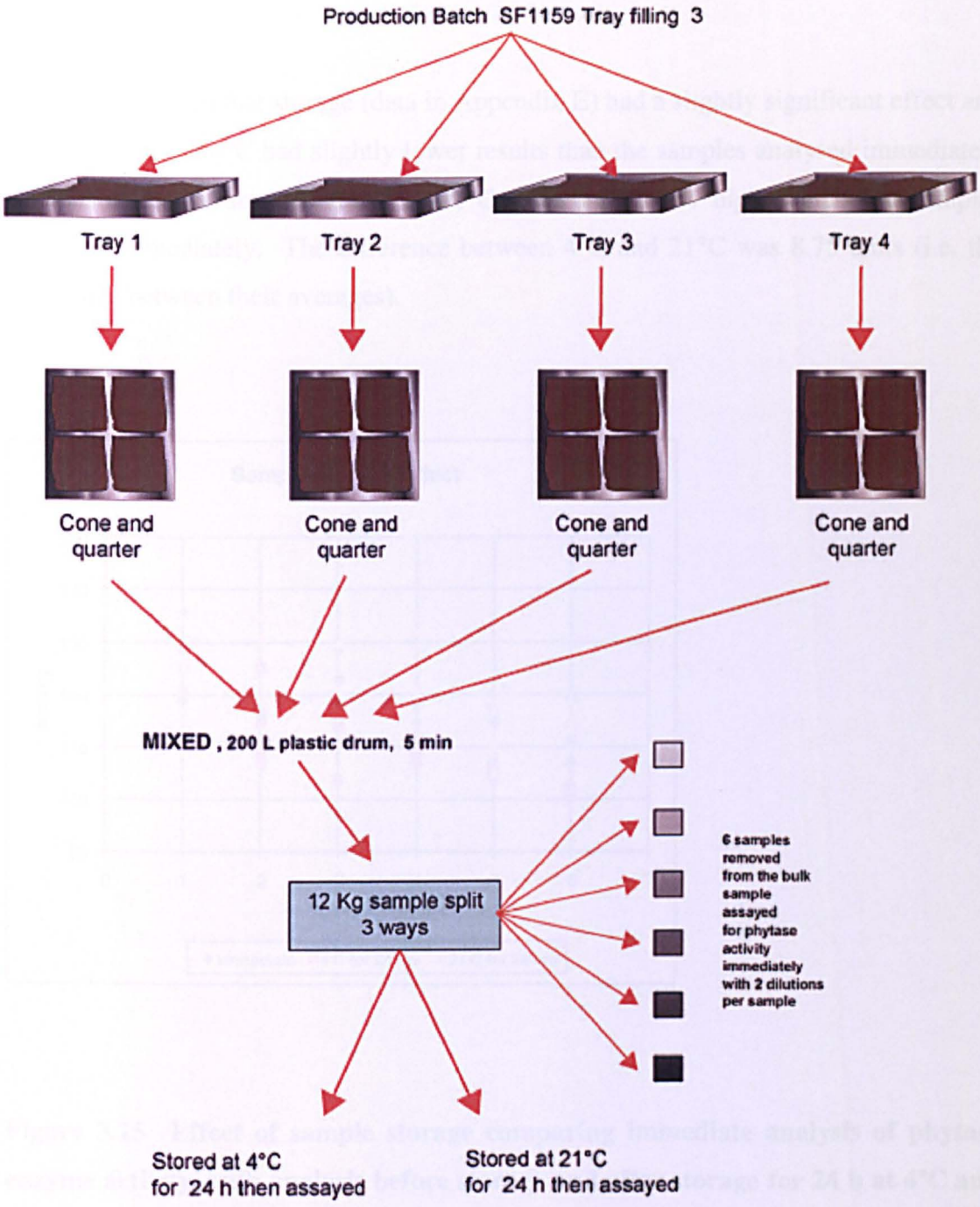


Figure 3.14 Sampling procedure for sample analysis storage experiment.

Four production trays from the same batch were selected, cone and quartered (Fig.3.14), six samples were stored at 4° C for 24 h and six samples were stored at 21° C for 24 h and then analysed in duplicate (with two dilutions for each sample) as one unit with the same batch of reagents following the methods outlined in Methods Section 2.11.2. One set of six samples was analysed immediately after cone and quartering and before storage.

The data indicated that storage (data in Appendix E) had a slightly significant effect and samples stored at 4°C had slightly lower results than the samples analysed immediately (-4.75), whereas samples stored at 21°C were 4.00 units higher than the samples analysed immediately. The difference between 4°C and 21°C was 8.75 units (i.e. the difference between their averages).

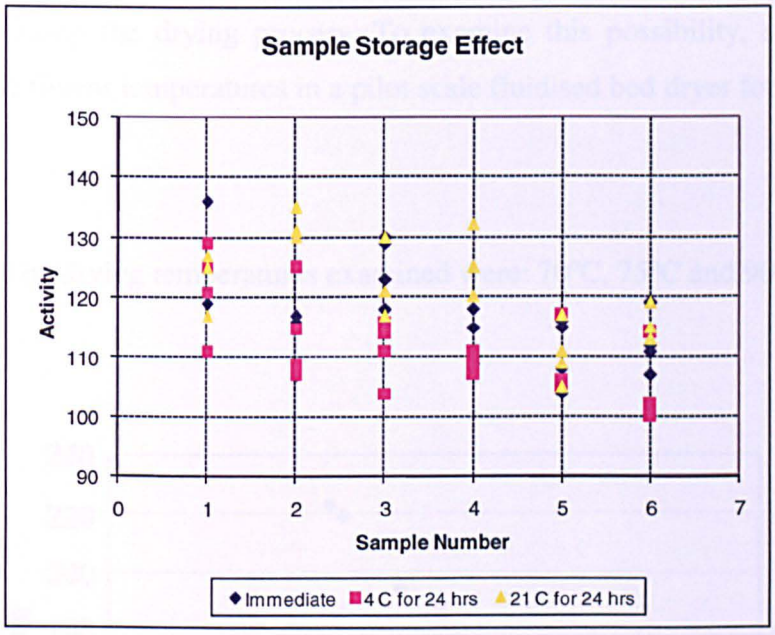


Figure 3.15 Effect of sample storage comparing immediate analysis of phytase enzyme activity, with analysis before storage and after storage for 24 h at 4°C and 21°C.

Based on the differences observed in Figure 3.15, the decision was made to ensure more consistency in sampling handling, and also as good manufacturing practice, samples being held for analysis are now stored at 4°C. If a sample should contain bacterial contaminants, holding the sample at the lower temperature before analysis would prevent their growth. Another concern had been that fungal cells might release some intracellular enzymes due to cell lysis and that these enzymes could interfere with the analysis, either positively or negatively. With the strain tested there was no large increase seen with the hold at 21°C for 24 h but this might occur with less sturdy strains

or species held over long time periods before analysis and this concern should be kept in mind when problems with analyses are detected.

3.3.3 Fluidised bed drying- effect of drying temperature

Koji samples, from production trays for phytase production, were routinely dried before they were cone and quartered and also before the product was shipped to customers. One of the causes of variation in final enzyme activity could be differences encountered during the drying process. To examine this possibility, samples were dried at three different temperatures in a pilot scale fluidised bed dryer for 20 min.

The drying temperatures examined were: 70°C, 75°C and 90°C.

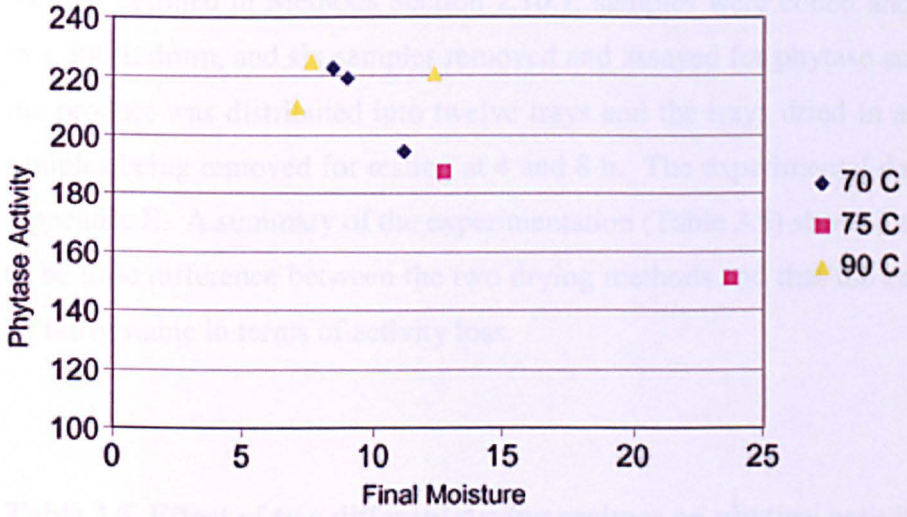


Figure 3.16 Phytase activity versus final moisture at three different drying temperatures.

A negative correlation was observed between final moisture and phytase enzyme activity i.e., the higher the final moisture the lower the activity (Fig. 3.16). It was not possible to say from this experiment if there was a cause-effect relationship. There was

no significant temperature effect over the range of temperatures studied (i.e. no up or down trend) suggesting that drying temperatures up to 90 °C for the drying procedure (at less than 20 min duration) were not detrimental to phytase activity in the final product.

3.3.4 Oven drying

To determine if there was a better way than fluidised bed drying to dry the large number of samples involved in large scale plant fermentation trials and to reduce variability in the drying process, attempts were made to use a drying oven where the trays could be inserted for drying, rather than a fluidised bed dryer. There was also interest in what was the effect on enzyme activity of drying over an extended time period.

Twelve trays were randomly selected from a fermentation chamber and following the method outlined in Methods Section 2.10.1, samples were coned and quartered, mixed in a 200 L drum, and six samples removed and assayed for phytase activity. The rest of the product was distributed into twelve trays and the trays dried in a hot air oven with samples being removed for testing at 4 and 8 h. The experimental data can be found in Appendix E. A summary of the experimentation (Table 3.5) showed that there appeared to be little difference between the two drying methods and that the enzyme appeared to be fairly stable in terms of activity loss.

Table 3.5 Effect of two different drying regimes on phytase activity.

	Fluidized bed drying	After 4 h of tray drying at 70°C	After 8 h of tray drying at 70°C
Enzyme Activity SPU/g	185 +/- 28	210+/-50	222 +/-20

3.3.5 Side activities

Although the prime enzyme of interest when using the *A. niger* strain was phytase, it has been clear from the start that side activities were numerous in this fermentation and that their existence would give this product its greatest value and unique nature. However, it was decided to standardise on the phytase activity as this was the most important of all the activities for feed applications. Henceforth, quality control of the product was based on this prime activity. However, studies were carried out to better understand the relationship between this main activity and the other coproduced side activities.

The activity of these strains was monitored for protease, cellulase, xylanase, and α-amylase activity over 90 plant scale fermentations. Fermentations were carried out with the standard plant scale parameters as detailed in Methods Section 2.8.2. The data was analysed to determine if there were correlations between the production of phytase and the other four specific enzymes. Table 3.6 shows the relationship between these side activities and their normal averages over the 90 fermentations.

Table 3.6 Side activities and their normal averages over 90 fermentations.

	Average	Std Dev
Phytase	410.41	51.75
Protease	1587.2	303.42
Alpha amylase	27.9	6.96
Cellulase	78.73	17
Xylanase	249.26	50.78
Phytase/Protease Ratio	0.27	0.07
Phytase/Alpha Amylase Ratio	15.55	4.14
Phytase/Cellulase Ratio	5.43	1.27
Phytase/Xylanase Ratio	1.72	0.45
Note: Units as defined in Methods Section 2.11.3		

Figures 3.17 to 3.20 compare each of the enzymes to phytase production in the same fermentation.

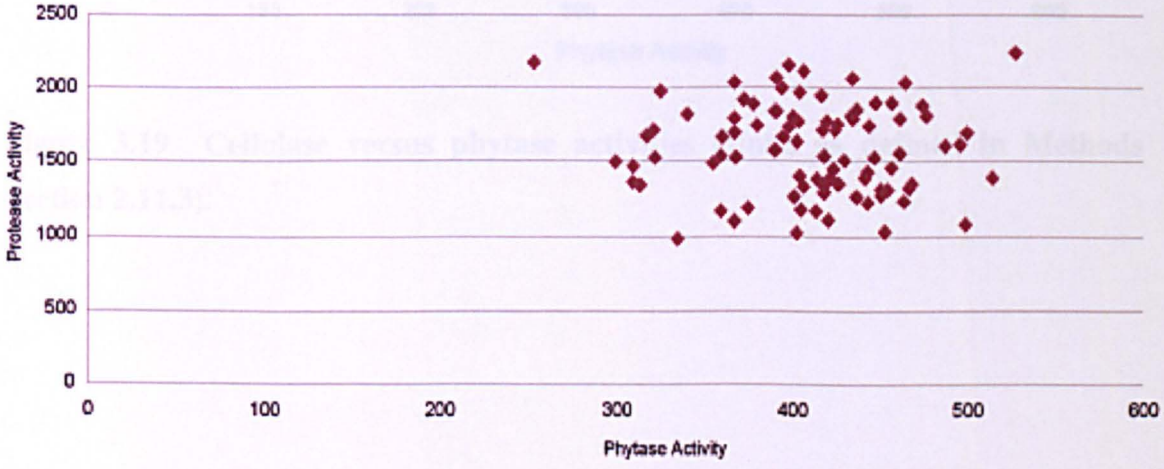


Figure 3.17 Protease versus phytase activities (Units as defined in Methods Section 2.11.3).

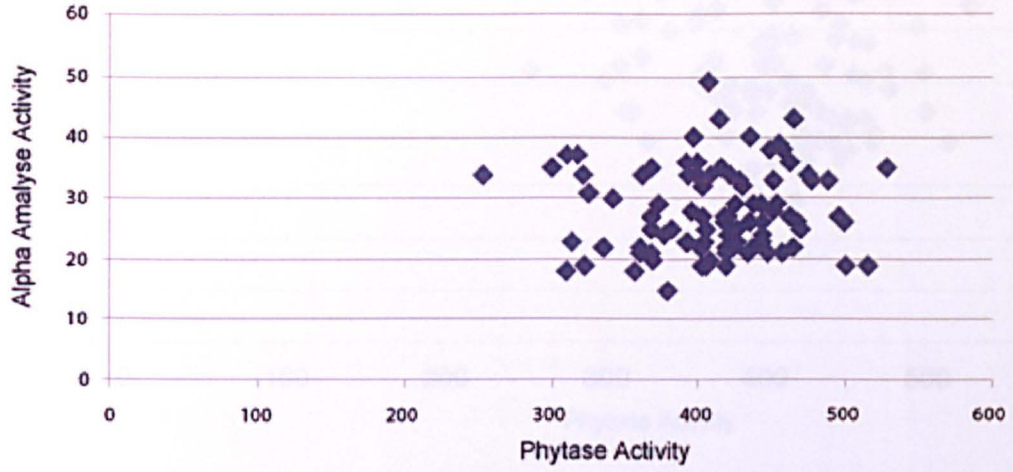


Figure 3.18 Alpha amylase versus phytase activities (Units as defined in Methods Section 2.11.3).

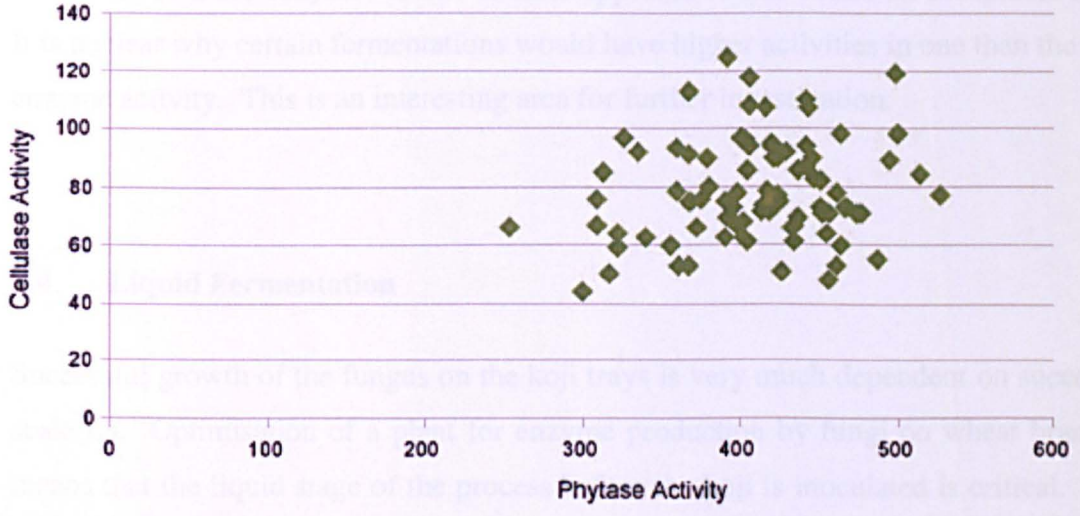


Figure 3.19 Cellulase versus phytase activities (Units as defined in Methods Section 2.11.3).

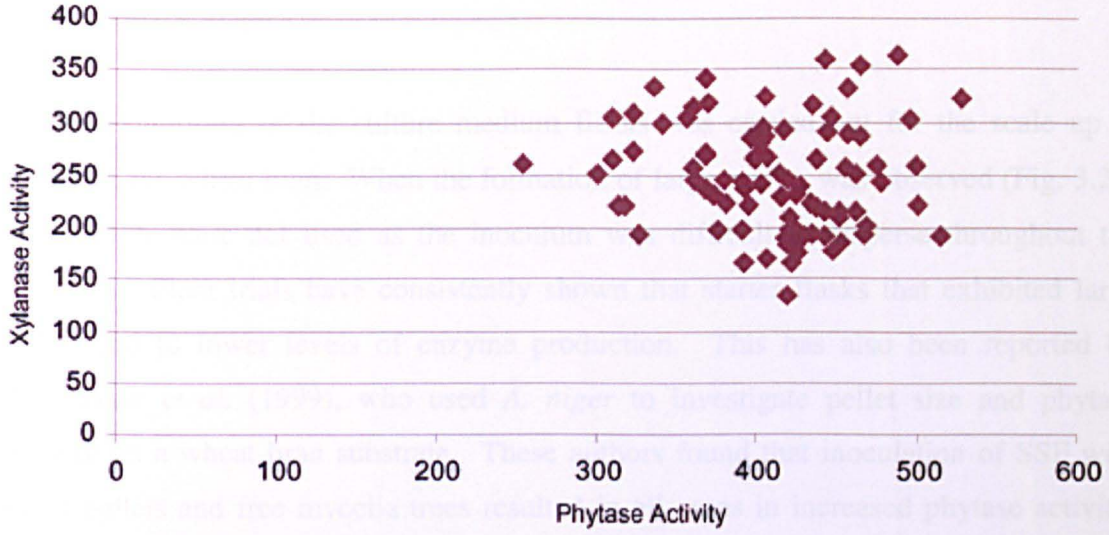


Figure 3.20 Xylanase versus phytase activities (Units as defined in Methods Section 2.11.3).

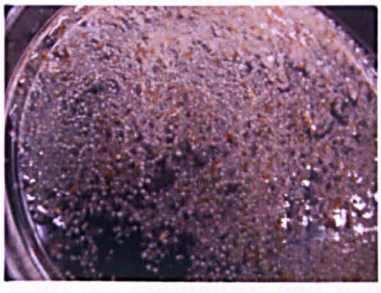
The ratios of these enzymes to one another appeared to fall within an acceptable range. It is unclear why certain fermentations would have higher activities in one than the other enzyme activity. This is an interesting area for further investigation.

3.4 Liquid Fermentation

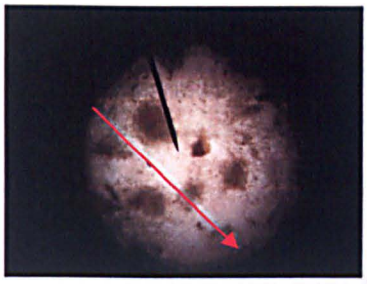
Successful growth of the fungus on the koji trays is very much dependent on successful scale up. Optimisation of a plant for enzyme production by fungi on wheat bran also means that the liquid stage of the process before the koji is inoculated is critical. Thus parameters that need to be considered include: the formulation of the liquid medium (nutrients and pH), temperature, agitation, the number of scale up steps required to obtain the volume needed for large scale inoculation, growth stage of the fungus in the scale up broth, and the dilution at which the bran is inoculated at the production scale i.e. how many fungal cells per gram of koji.

3.4.1 Liquid media

Visual monitoring of the culture medium flasks was carried out for the scale up to inoculate the wheat bran. When the formation of large pellets was observed (Fig. 3.21) these flasks were not used as the inoculum was difficult to disperse throughout the substrate. Plant trials have consistently shown that starter flasks that exhibited large pellets led to lower levels of enzyme production. This has also been reported by Papagianni *et al.* (1999), who used *A. niger* to investigate pellet size and phytase activity on a wheat bran substrate. These authors found that inoculation of SSF with small pellets and free mycelia trees resulted in all cases in increased phytase activity, and that large pellets were not suitable as a morphological form for SSF inoculation, unless they were first homogenised. Once homogenised, these pellets gave similar productivities to those from filamentous mycelia, however homogenising large quantities of starter culture is not a practical or cost effective operation. Thus visual screening, to avoid cultures that formed large pellets, was implemented. With large pellets, growth in the centre is hindered by nutrient transfer limitations. Depending on the metabolite in some cases, formation of mycelia pellets is what is desired to produce fungal enzymes such as α-glucosidase, whereas other enzymes such as phytase have higher levels when the free filamentous morphology is present.



Flask A



Flask B

Flask C

Figure 3.21 Photograph illustrating *A. niger* after growth in S2 medium (200 ml flask) for 72 h growth on a shaker table (200 RPM) at 30°C. Flask A shows large pellet formation in the flask. Flask B is the same sample but at a magnification of x 4.1. Flask C illustrates typical desirable flask growth with only very small pellets.

Experiments have been conducted, both at the plant in Serdán Mexico, and at Alltech's European Bioscience Centre in Dunboyne Ireland, examining dilution steps in the early stages of liquid culture (S2). This research was not part of this project. Their data from both the laboratory and plant trials indicated that a dilution factor of 1:20 was ideal. This was especially important for the phytase fermentations with *A. niger,* where the strain was not as aggressive in terms of growth as the *A. oryzae* strain. This dilution was better at preventing contaminants from gaining a foothold on the koji trays. Consequently, a 1:20 dilution was accepted as the routine procedure for inoculation of the organism into the S3 stage.

3.4.2 Inoculation method (liquid media)

In terms of standardisation of the flask experiments, a consistent inoculum from the liquid stage onto the solid koji was a key success factor. Experiments were conducted that examined how best to obtain consistent inoculation in the koji flasks, since it was very difficult to remove equivalent amounts from the liquid flask starter culture to ensure that each koji flask had the same inoculum. Mixing a large batch of koji with the liquid and then allocating it, as is the practice in the production plant, was not feasible when flasks rather than trays are used.

S2 medium containing *A. niger* was used to inoculate the koji at a 1:20 dilution to see if variability in the analyses was due to the method of inoculation (Table 3.7). Duplicate flasks were shaken vigorously by hand, or gently agitated with a magnetic stirrer or mixed with a sterile pipette and then the required aliquot was removed. Analysis for phytase enzyme was carried out after growth for four days at 30° C on the wheat bran in the koji flask.

Table 3.7 Inoculation methods from S2 to koji flasks for phytase production.

Mixing methods:	Replicates	Set 1 (SPU/g)	Set 2 (SPU/g)	AVERAGE:
A. Flask contents mixed with sterile pipette*	Flask 1	634	624	579 +/-58
	Flask 2	533	526	
B. Flask contents shaken vigorously before inoculum was pipetted	Flask 1	597	590	585 +/-39
	Flask 2	624	621	
	Flask 3	529	547	
C. Flask contents shaken lightly before inoculum was pipetted	Flask 1	502	465	473 +/-21
	Flask 2	488	477	
	Flask 3	462	441	
D. Magnetic stirrer used for 3 min before inoculum was pipetted	Flask 1	617	578	613+/-21
	Flask 2	635	611	
	Flask 3	607	629	

* Flask 3 samples lost due to breakage

The sampling method showed good consistency with the exception of flask C, the flask that was not vigorously shaken. This would be expected as there may not have been enough agitation to properly mix the inoculums. Nevertheless, to standardise the inoculation procedure for koji flask experiments, a standard procedure was adopted using a magnetic stirrer to keep the fungal mycelia in the starter flask well mixed before pipetting into the koji flasks. However, this particular procedure did not appear to be a large source of error unless there was a presence of pellets. When heavy pellets were present, the flask was not used for inoculation and new starter cultures were cultivated.

3.5 SSF

SSF faces challenges very different from liquid fermentation. Sourcing the bran, ensuring it is the correct particle size and evaluating the depth of the bran bed are all areas that are not a concern in liquid fermentation. Chamber location is another parameter that is extremely difficult to deal with and is not encountered in liquid fermentation.

3.5.1 Effect of wheat bran sources

Wheat bran is a secondary product of the flour production process and as a result the particle size, as well as the nutritional value, will vary depending on the supplier and the specific process and the batch lot. As an agricultural product, the nutrients in it will also vary with environmental growth conditions of the wheat. All of the wheat bran utilised in this project was from one supplier with the exception of this study. Experiments were carried out to examine if a different supply of wheat bran would affect fungal growth for phytase production.

Both the production incubation chambers as well as the koji flask procedure were employed and the standard techniques outlined in Methods Section 2.8.1 were employed.

Analysis sheets for the three wheat bran products can be found in Appendix C.

Three plant trials using three different incubation chambers and starter inoculum batches were employed to investigate whether the supplier of the wheat bran affected the phytase activity level.

Table 3.8 Phytase activities (SPU/g) on a wet and dry basis for three production batches incubated on separate days and in separate fermentation chambers.

Batch 1				
Bran Supplier	Phytase activity SPU/g (Wet basis)			Average phytase activity SPU/g in dried product
	Day 2	Day 3	Day 4	Day 4
San Blas	60	190	139	343
La Asuncion	59	182	314	458
El Paraíso	23	81	217	246
Batch 2				
Bran Supplier	Phytase activity SPU/g (Wet basis)			Average phytase activity SPU/g in dried product
	Day 2	Day 3	Day 4	Day 4
San Blas	42	145	162	490
La Asuncion	20	166	376	466
El Paraíso	11	117	220	412
Batch 3				
Bran Supplier	Phytase activity SPU/g (Wet basis)			Average phytase activity SPU/g in dried product
	Day 2	Day 3	Day 4	Day 4
San Blas	30	139	344	496
La Asuncion	10	84	306	446
El Paraíso	34	33	147	359

Results in Table 3.8 indicated that there were differences between the three suppliers with the El Paraíso wheat bran consistently showing the lowest phytase activity.

Differences in the wheat bran are not surprising when they are from different manufacturers who vary in how much flour is extracted from the bran, which

consequently affects the nutritional value of the substrate.

3.5.2 Particle size

Particle size in wheat bran is a very important variable to be considered in SSF. Too small particles may lead to packing, and large particles may not offer a good surface for microbial growth. A balance between small and large particles is believed to be essential to achieve good fermentation (Mitchell et al., 2006).

In order to determine whether or not particle size played an important role in the stimulation of enzyme production, the following analysis was carried out. Five kg of a wheat bran sample was subjected to a sieve analysis in which each fraction was retained. Subsequent to this, sterile 10 g samples of each fraction were inoculated into growth medium and incubated as described in Methods Section 2.8.1. For each bran fraction, four flasks were inoculated and analyses were conducted in duplicate. The average activity produced from each fraction can be seen in Table 3.9. Both the very small particles < 150 micron and the very large base particles when separated and inoculated, yielded the poorest phytase activity.

Table 3.9 Wheat bran separated by size and inoculated with *A. niger* for phytase production in koji flasks at 30°C.

Sieve mesh #	Particle size range (microns)	Protease activity (HUT/g)
14	>1400	8,420 ± 2,153
18	1000 – 1399	8,242 ± 1,008
35	500 – 710	8,069 ± 804
80	180 – 250	7,057 ± 1,210
Control	Not sieved	8,012 ± 1,598

Particle size in SSF is an important parameter. A too small particle size will lead to packing of the bed and too large a particle size will not offer sufficient surface area for microbial growth. Thus a balance is needed between the two extremes.

A similar experiment was carried out looking at protease activity using *A. oryzae* and it appeared that as with the *A. niger* strain, particle size in the range examined was not an important variable.

Smaller substrate particles provide a larger surface area for the microbial action and for a constant geometry, the surface area to volume ratio increases as the particle size decreases. Smaller particles are also advantageous for heat transfer and for the exchange of oxygen and carbon dioxide between the air and the solid bran particle. The size of the substrate particle determines the void space and the rate of oxygen transfer into the void space affects growth. The goal is to have particles of such a size as to enhance mass transfer. It must also be remembered that the substrate is consumed by the fungus during the fermentation, thus the size of the particles do not stay constant as the quantity of the bran is reduced during the fermentation.

3.5.3 Effect of bran depth on phytase activity

Numerous plant trials investigating bed depth have been carried out (data not included).

The goal was to optimise plant productivity (maximum enzyme production with minimum time). Although increasing the amount of koji per tray yields more final product i.e. grams per tray, this may not translate into more enzyme activity per gram in that final product.

One typical set of experiments is given as an illustration of the effect of bed depth.

Historical plant trials have indicated that a height of 2.5 cm in the tray yields acceptable

results. The two experiments illustrated below were carried out on different days using a bran test height of 2.5 cm (normal production height) and 5.0 cm (test height).

Effect of bran height in the trays was tested using a bran control height of 2.5 cm (normal production height) and a test height of 5 cm. Two separate experiments (Tray filling A - trial one and Tray filling B - trial two) were carried out. The bran substrate, method of inoculation, production trays and incubation conditions in the chambers as well as the *A. niger* strain were standard as described in Methods Sections 2.4 – 2.6. Phytase activity was determined as described in Methods Section 2.11.2 and was expressed on both a wet and dry basis. All analyses were carried out in triplicate. Increasing the depth of the wheat bran in the trays had a negative effect on phytase activity from *A. niger* (Fig. 3.22).

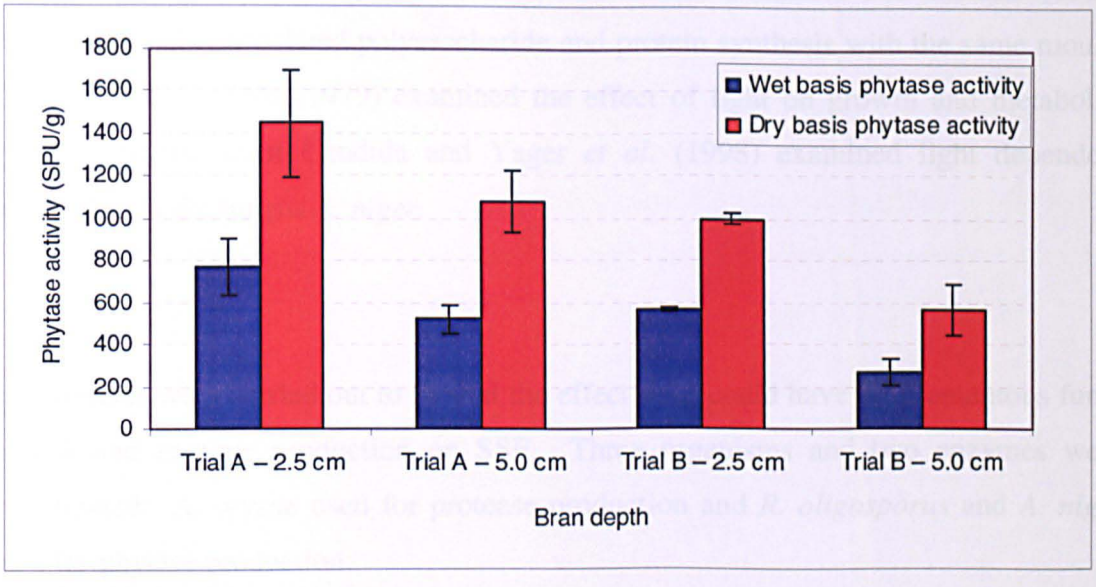


Figure 3.22 Effect of bran depth on phytase production by *A. niger* in koji trays after four days fermentation under standard plant conditions.

Extensive research was carried out by Rajagopalan and Modak (1994) in terms of modelling bed depth and Ikasari and Mitchell (1998) measured temperatures as high as 50°C at a 5 cm depth in a *R. oligosporus* wheat bran fermentation. Mitchell *et al.*

(2006) suggest that trays are limited to a bed height of around 5 cm due to issues of heat and oxygen transfer within the bed. Although the substrate, the specific organism and the environmental conditions will all influence the ideal bed height, past work in the production plant in Serdán, in terms of optimisation, is in agreement with the suggestion that a height of 5 cm would be the maximum that can be utilised in a production plant without large losses in enzyme activity. Increased depth of bran in the trays allows more throughput of wheat bran during the same time period, but the gains in throughput must be balanced against lower enzyme production.

3.5.4 Light effects

The effect of light on *A. niger* and *A. oryzae* in solid culture has not been well studied to date. However, light effects on other fungi have been well documented. For example, Turian and Cantino (1959) reported on the stimulatory effect of light on nucleic acid synthesis in the mould *Blastocladiella emersonii* and Goldstein and Cantino (1962) reported on light-stimulated polysaccharide and protein synthesis with the same mould. Saltarelli and Coppola (1979) examined the effect of light on growth and metabolite synthesis in the yeast Candida and Yager *et al.* (1998) examined light dependent conidiation in the fungus *A. niger.*

Experiments were carried out to look at the effect light could have on filamentous fungi growth and enzyme production on SSF. Three organisms and two enzymes were investigated: *A. oryzae* used for protease production and *R. oligosporus* and *A. niger* used for phytase production.

Koji flasks were prepared according to standard procedure detailed in Methods Section 2.8.1. Incubation was carried out at 30°C for three days with *A. oryzae* and for four days with *R. oligosporus* and *A. niger.* All experiments were performed in triplicate and the results are reported on a wet koji basis.

One set of koji flasks were wrapped with aluminium foil to prevent light from entering the flasks and one set was left unwrapped and exposed to the light from a 100 watt incandescent naked bulb. As can be seen on Table 3.10, lack of light did not appear to have an effect on protease production by *R. oligosporus.* However, both of the two Aspergillus species tested appeared to respond to the light, with *A. oryzae* producing 16% more protease and *A. niger* producing 27% more phytase in the presence of light.

Table 3.10 Effect of light on protease and phytase production in koji flasks at 30°C.

Culture	Enzyme Activity of Flask Kept in Dark	Enzyme Activity of Flask Exposed to Light
Aspergillus oryzae	34807 ± 2648 HUT/g	40592 ± 2591 HUT/g
Rhizopus oligosporus	11156 ± 2163 HUT/g	11405 ± 2579 HUT/g
Aspergillus niger	571 ± 6 SPU/g	727 ± 13 SPU/g

These preliminary results require further experimentation to confirm this effect but the experiment showed some promise. Since the koji trays for large scale production are all covered with lids, experimental work was not further pursued for the time being. Opportunities could exist in the future to work with uncovered koji trays using another type of cooling or perhaps using a bioreactor. In this case, the effect of light on increasing enzyme production could be one more useful optimisation tool. It may also be considered in terms of a standardisation tool. Light may be a factor that needs to be kept consistent when carrying out experimentation with koji flasks, which may or may not be exposed to light during laboratory fermentations, depending on the mode of incubation (i.e. incubation room with lights versus dark incubator).

3.5.5 Chamber location effects

One of the unknown variables in the production system focuses around differences in

temperature, air flow, and gas transfer in the chambers. Since the chambers are very large, it is clear that location in the chamber will have an effect on each individual tray. Whether the tray is located under a spray head in terms of access to moisture, close to an air removal duct, close to a wall or in the centre of the incubation chamber, all will affect the microenvironment of the tray. In addition, its location on the trolley will also influence the microenvironment. Is the tray on the top of the trolley and thus has maximum ability to take advantage of cooling in the room from the water spray or is the tray in the middle rack of the trolley with maximum exposure to heat produced from other trays on the trolley located above and below the selected tray? Even selecting a tray for analysis during the course of an experiment will affect the environment. Since the testing process is destructive, a tray can never be sampled twice, so adjacent trays must be selected for the next sample. A series of experiments was conducted to look at the process over a two month period.

The periods have been labelled April and May on the figures and the goal was to look at whether the observed differences i.e. the averages, seen in the production chambers, were varying due to chance alone (i.e. measurement) or was there a real difference in the product (i.e. process).

Three areas were examined and analysis of variance was used to break the results of the nested design into components of variance, both among trays, among samples within each tray, and among tests within each sample.

Averages of phytase enzyme production are illustrated in Table 3.11.

Table 3.11 Averages of phytase enzyme production in koji trays in production fermentation chambers.

Tray No.	Phytase Average SPU/g		The % of overall Average*	
	April	May	April	May
1	101 SPU/g	92 SPU/g	75%	80%
2	141 SPU/g	120 SPU/g	105%	104%
3	179 SPU/g	155 SPU/g	133%	134%
4	117 SPU/g	95 SPU/g	87%	82%
Overall	135 SPU/g	116 SPU/g	100 %	100 %

*Removal of the block effect due to testing at different times.

What was observed was that the measurements could discriminate among the trays. In terms of ranking, both sets of test results (April and May) ranked the trays 1, 4, 2 and 3 in ascending order (lowest to highest enzyme levels). In terms of quantitative, although 'overall averages' were significantly lower for May (14%), there was good agreement among test results, when each was expressed as a percentage of its overall average (i.e. after removing the block effect due to testing at different times). For example, Tray 3 contained 33% to 34% more enzyme than the average for the four trays whereas Tray 1 only contained 75% to 80% of the average. The conclusion that could be drawn was that when the trays were tested at the same time, it proved to be a very good measurement tool to discriminate among enzyme levels. However, the observed difference in overall averages between the April and May samples still needed to be explained.

Looking at the process in terms of tray-to-tray variability carried out using analysis of variance in the nested designs for April and May and this data showed excellent agreement with a SIGMA for April of 33 units and SIGMA for May of 31 units and the average SIGMA for all trays tested of 32 units. Thus, the differences in the tray averages were not explained by chance alone; i.e. the trays were different. The process (tray-to-tray variability) represented the largest portion of variability. With a very large

number of "cone and quarter" samples and many assays per "cone and quarter" sample the sensitivity analysis was as illustrated in Table 3.12.

Table 3.12 Sensitivity analysis on phytase production by *A. niger*.

Number of trays selected	*1*	*4*	*8*	*16*
Difference among trays detectable (units)	*91*	*45*	*32*	*23*

With eight trays selected from each tray filling batch, one could be 95% certain that a difference as large as 32 units would be found. Any difference less than 32 units would be considered as the same or no difference. This was based on a SIGMA estimate from adjacent trays, and this estimate may have been low.

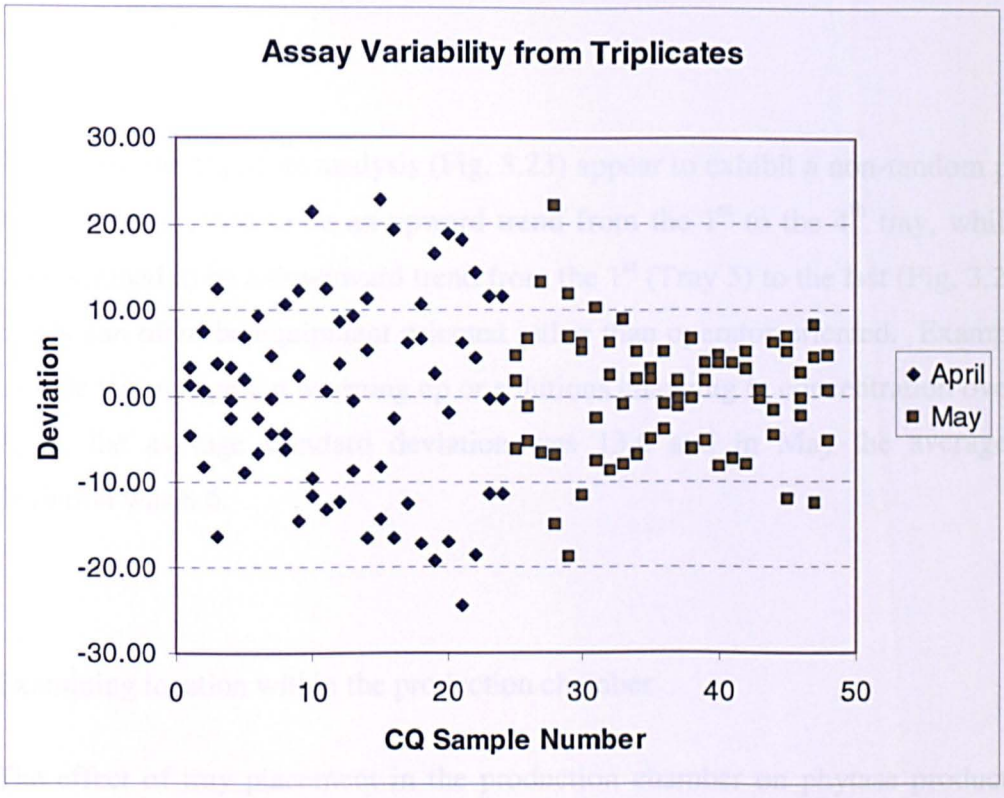


Figure 3.23 Phytase assay variability from triplicates (CQ = cone and quarter).

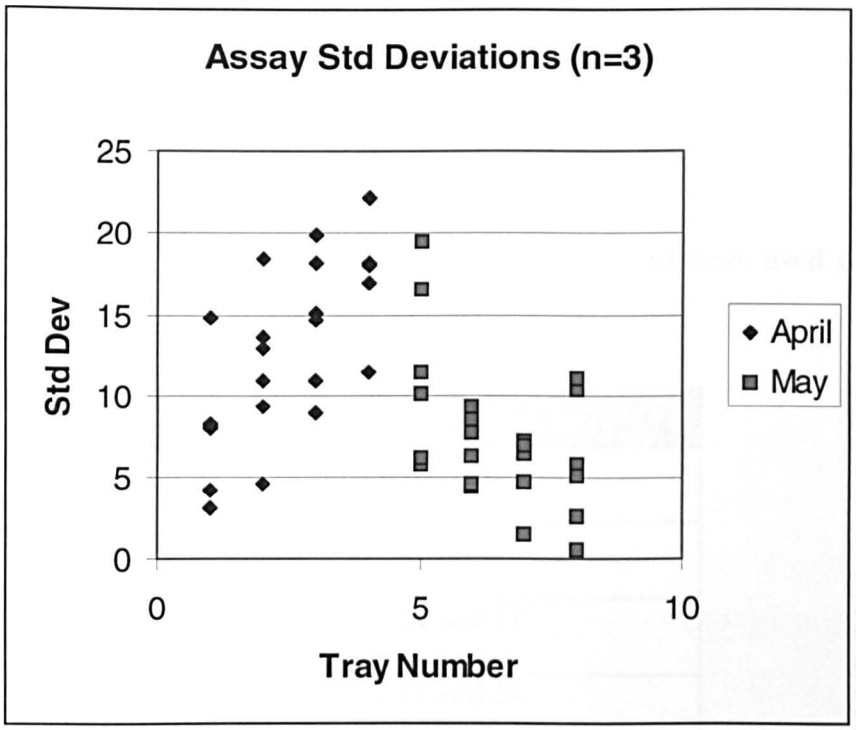


Figure 3.24 Phytase assay variability based on tray.

Results for the triplicate analysis (Fig. 3.23) appear to exhibit a non-random pattern. In April, there seemed to be an upward trend from the 1st to the 4th tray, while in May, there seemed to be a downward trend from the 1st (Tray 5) to the last (Fig. 3.24). These trends can often be equipment oriented rather than operator oriented. Examples would include test equipment warming up or solutions changing in concentration over time. In April, the average standard deviation was 13.6 and in May the average standard deviation was 8.6.

Examining location within the production chamber

The effect of tray placement in the production chamber on phytase production by *A. niger* was examined. One batch of liquid inoculation of *A. niger*, produced using standard plant production techniques as described in the Methods Section 2.6.2, was used to generate five tray filling batches (i.e. the same starter batch of fungus was used to inoculate five batches of wheat bran sequentially). This was enough koji to fill 30 production trolleys. The trolleys were placed in Chamber two as shown in a previous

lay-out and two trays (22 and 25) were taken from twelve trolleys selected from the possible 30 trolleys as described in Table 3.13.

Table 3.13 Tray filling batches and trolley numbers used to select the trays for analysis.

Tray Filling (TF) Batch	Trolley Number
TF 1	1, 2 and 5
TF 2	7 and 12
TF 3	14 and 17
TF 4	19 and 24
TF 5	26, 29 and 30

The test results for six samples (A and B analysis) per tray were used to estimate the *within-tray variability*. This factor was a $SIGMA_{within\text{-}tray}$ of 10.3 units (see Table 3.14). This allowed for the detection of differences as small as 26 units, 95% of the time between two trays and in this case between tray 22 and tray 25. The difference of 27 units for trolley 26 can be explained by chance only 2 in 1000 times.

Table 3.14 Estimate of within-tray variability using six cone and quarter samples per tray.

Trolley	Tr25- Tr22 Difference	SS (df) 11 Total	1 Regression	10 Residual	MS Regression	Residual	F-ratio	Signif	Within - Tray Variability
1	-60	10940.56	10591.02	349.54	10591.02	34.95	303.00	0.000	5.9
2	76	17880.50	17176.33	704.17	17176.33	70.42	243.92	0.000	8.4
5	6	1435.25	126.75	1308.5	126.75	130.85	0.97	0.348	11.4
7	183	102052.17	99918.75	2133.42	99918.75	213.34	468.35	0.000	14.6
12	-100	30377.75	30000.00	377.75	30000.00	37.78	794.18	0.000	6.1
14	-99	30564.73	29254.69	1310.04	29254.69	131.00	223.31	0.000	11.4
17	-80	21014.23	18841.69	2172.54	18841.69	217.25	86.73	0.000	14.7
19	86	22731.67	22274.08	457.58	22274.08	45.76	486.78	0.000	6.8
24	-149	67428.73	66677.52	751.21	66677.52	75.12	887.60	0.000	8.7
26	27	3437.73	2227.69	1210.04	2227.69	121.00	18.41	0.002	11.0
29	76	17932.75	17404.08	528.67	17404.08	52.87	329.21	0.000	7.3
30	-165	82953.73	81592.52	1361.21	81592.52	136.12	599.41	0.000	11.7
Pooled Average (obtained by pooling Residual Sum of Squares (SS):									10.3

The Tray-to-Tray Variability was estimated from each pair of trays on the same trolley (Table 3.14). Using pairs from the "same" location ensured that a possible chamber location effect was minimised. Table 3.15 shows the Day 4 phytase activities.

Table 3.15 Day 4 phytase activity (SPU/g) per tray. Average of six cone and quarter samples.

	Trolley Number											
	1	2	5	7	12	14	17	19	24	26	29	30
Tray 22 (SPU/g)	261	183	272	291	385	456	474	130	273	195	252	337
Tray 25 (SPU/g)	201	259	278	474	285	357	394	216	124	222	328	172
Differences Tray 25-22	-60	76	6	183	-100	-99	-80	86	-149	27	76	-165

The SIGMA $_{\text{tray-to-tray}}$ was 74.17. Figure 3.25 illustrates the reproducibility of results from tray-to-tray at the same physical location in the chamber. The amount of scatter (at right angles to the centre line) indicates the magnitude of the existing variability. The distance of each point was determined by the absolute difference between the two results. For example, the point corresponding to trolley 7 was +183/Sqrt(2) = 129 perpendicular units to the left of the line. Similarly, the point for trolley 30 was -165/sqrt(2) = -117 perpendicular units to the right of the line. The estimated 2σ prediction limits were approximately 150 perpendicular units. The width of this perpendicular band represents noise whereas the differences along the line represent process changes or signal (e.g. tray filling 3 vs. tray filling 4).

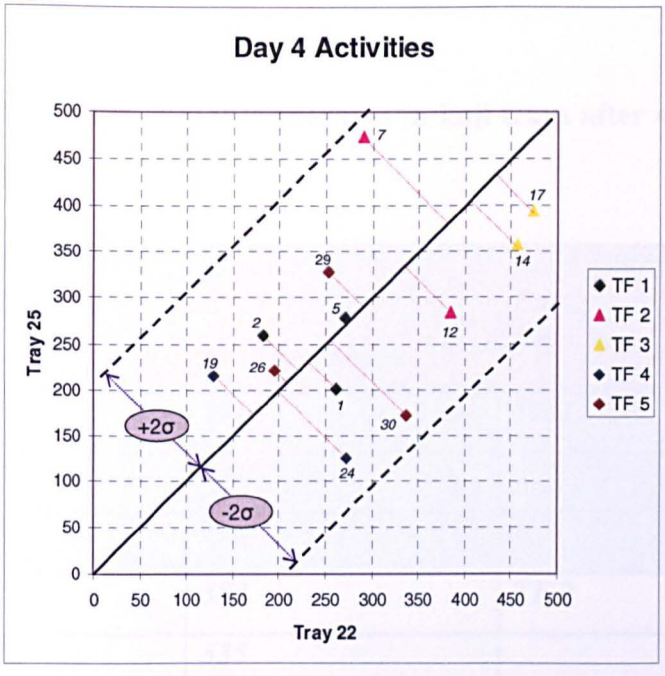


Figure 3.25 Reproducibility of results from tray-to-tray at the same physical location in the chamber over five different tray fillings.

Trolley-to-trolley and batch-to-batch differences were also examined and the phytase tray averages shown in Table 3.16.

Table 3.16 Phytase averages in koji trays after 4 days of incubation in production chambers.

Trolley Number	Phytase (SPU/g) *Trolley Average*	*Tray Filling Batch Number*	Phytase (SPU/g) *Tray Filling Average*
1	*231*	*TF 1*	*242*
2	*221*		
5	*275*		
7	*383*	*TF 2*	*359*
12	*335*		
14	*407*	*TF 3*	*420*
17	*434*		
19	*173*	*TF 4*	*186*
24	*199*		
26	*209*	*TF 5*	*251*
29	*290*		
30	*255*		

In was important to see if trolley placement order in the chambers had an effect on the phytase production. Day four activities were examined (Figure 3.26).

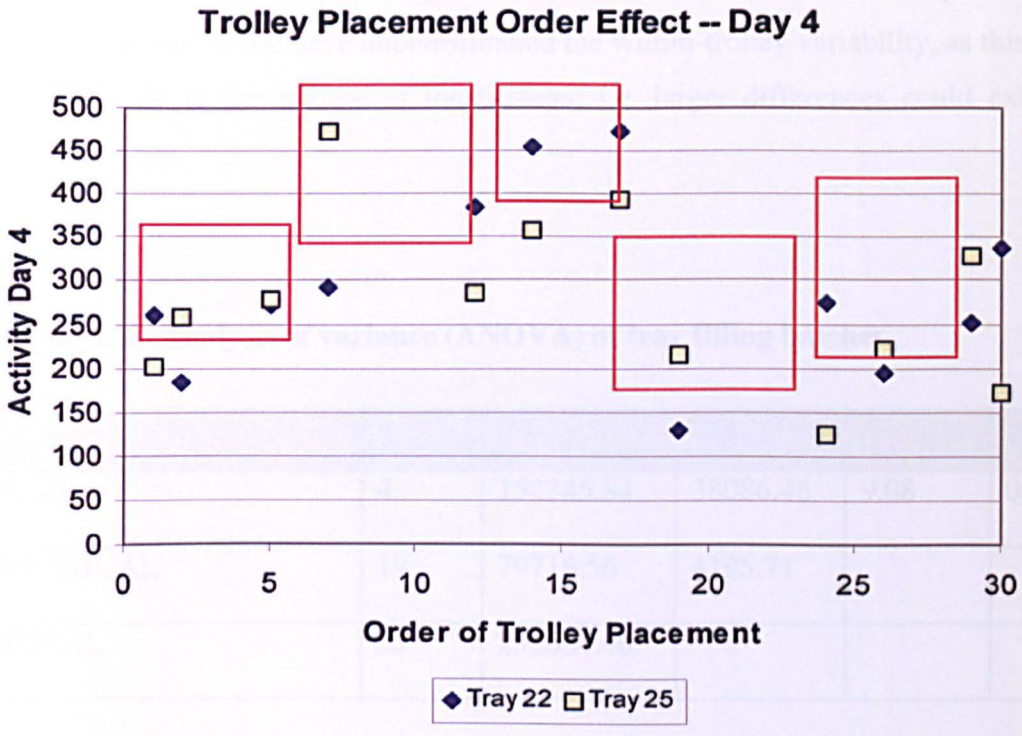


Figure 3.26 Phytase activity at day 4 and trolley placement in incubation chamber.

In Figure 3.26, each red rectangle represents a tray filling batch. The analysis of variance (ANOVA) showed significant differences between trolley (Table 3.17) and tray filling batch averages (Table 3.18).

Table 3.17 Analysis of variance (ANOVA) trolley-to-trolley of phytase production by *A. niger*.

	df	SS	MS	F - ratio	Significance
Trolleys	11	166050.21	15095.47	2.74	0.048
RESIDUAL	12	66014.19	5501.18		
TOTAL	23	232064.40			

The Standard Error = Sqrt (5501.18) = 74.17 estimated the tray-to-tray variability. It was used in this case as an estimate of the within-trolley variability to compare differences among trolleys. If significant differences existed from the top of a trolley to the bottom this would have underestimated the within-trolley variability, as this estimate applied only to the middle of the trolleys; i.e. larger differences could exist at the bottom or top.

Table 3.18 Analysis of variance (ANOVA) of tray filling batches.

	df	SS	MS	F - ratio	Significance
Trolleys	4	152345.84	38086.46	9.08	0.0003
RESIDUAL	19	79718.56	4195.71		
TOTAL	23	232064.40			

Table 3.18 indicates that 81% of the total variability could be explained by the five tray filling batches. The residual (unexplained portion of the TOTAL variability) consists of two portions as shown below in Table 3.19. The five tray filling batches contributed to the portion as follows: TF 1:3 trolleys per batch – therefore, 2 degrees of freedom (df) and TF 2:2 trolleys per batch – therefore, 1 df, etc. The trolley variability within the five batches was small compared to tray-to-tray within each trolley (i.e. statistically it was not significant).

Table 3.19 Analysis of residual variability.

	df	SS	MS	F - ratio	Significance
Trolleys within batch	7	13704.37	1957.77	0.36	Not significant
RESIDUAL	12	66014.19	5501.18		
TOTAL	19	79718.56			

Summary of the chamber location effects is as follows:

The SIGMA $_{\text{within-tray}}$ equalled 10.3 units. Repeat measurements on each tray allowed detection of real tray-to-tray differences. The sensitivity at six cone and quarter samples (two analyses each) reflected a 26 SPU/g difference between two trays.

The SIGMA $_{\text{tray-to-tray}}$ equalled 74.17 units. By taking measurements on two trays physically close together in the chamber one could detect real differences among the trolleys. Unfortunately, the current tray-to-tray variability was very large compared to the batch-to-batch differences and possible location differences within the chamber.

Based on this analysis, although the preference is always to do trials on a plant scale rather than in koji flasks, due to the issues of scale up discussed earlier, until the source of the large tray-to-tray differences can be found and eliminated, the result of treatment effects will need to be studied in flasks. This is in order to have confidence that these effects are in fact statistically significant before attempting large scale trials. Determining the source of the large tray-to-tray differences will be beneficial to understanding the tray filling process. For example, any other measurements on the trays that would help to explain the current tray-to-tray variability could be used to reduce the variability. This would increase the sensitivity of any experiments conducted in the chambers.

3.6 Single Variable Optimisation Experiments

Trials were carried out looking for improvements in enzyme yields and fermentations that were more efficient. Initially, one variable was changed at a time in order to look for potentially interesting factors that created some improvement in the performance of the microorganisms.

Single variable testing, commonly referred to as A/B testing, is a widely used form of testing. Modifying one variable, while keeping all others the same, theoretically allows for the assessment of one variable on its own in a complex multi-factorial design and thus one can evaluate whether it is important or not, and to what degree. Modifying one variable at a time is a rule of thumb in many industrial systems that deal with a significant level of unknown or uncontrollable variation due to their size and complexity.

As with any testing system, there are advantages and disadvantages to single variable experimentation. Advantages include the ease of designing experiments, the simplicity and clarity in explaining results, and the ease of implementation and utilisation of those results. Disadvantages can include the limitation of using one variable at a time, the need to increase the number of trials conducted in order to gain complete information about the subject variable being analysed, and the possibility of missing a more full understanding of the experimental system by looking at only part of it.

For the purposes of this study, single variable experimentation and optimisation was used to orient the analysis and increase the knowledge of the factors that could be important for the organism. Two general approaches were taken, modifying the nutrient levels of the organism through supplementation and fermentation parameter manipulation.

3.6.1 Wheat bran and nutrient supplementation

Supplementation of the fermentation medium is a standard way to look for improvements in fermentation performance. In SSF, the medium used is often fibrous and complex. Nutrient supplementation for SSF has not been looked into as extensively as in submerged fermentation, where it is standard and required. The solid nature of the material also means that mixing can be a challenge and therefore supplementation adds some difficulty to the process. However, the potential benefits to being able to induce a significant increase in enzymatic activity outweigh the difficulty of the task. Therefore, a number of materials that could be added to the wheat bran, both feasibly and economically on a production scale, were investigated and experiments to see if they augmented the fermentation ability of the production SSF strains were carried out.

3.6.1.1 Carbon and nitrogen

A logical starting point for supplementation was seen to be the two basic elements, carbon and nitrogen, both building blocks of organic life. Cellular biomass presents an average of 40-50% carbon, 30-50% oxygen, 6-8% hydrogen and 3-12% nitrogen (Pandey *et al.*, 2001a). After carbon as an energy source, nitrogen is the next most crucial factor that influences the growth of microorganisms. Elements such as phosphorus, sulphur, and metals are also important, but are required in much smaller quantities.

Carbon would be considered to be at sufficient levels in the wheat bran. Trials carried out in Alltech's European Bioscience Centre (Dunboyne, Co. Meath, Ireland) showed that addition of other carbon sources did not improve phytase production by *A. niger* on wheat bran. Mannose was the only sugar added that had a small positive effect on phytase production and only at the higher addition level. Their findings are listed in Table 3.20 and are reported on a percentage basis compared to the control, which was grown on wheat bran without any supplementation. Due to this research and other

similar experiments conducted in Ireland, where no significant benefit of simple sugar supplementation of wheat bran supplementation could be observed, the scope of this study did not include additional experiments in this specific area.

Table 3.20 Effect of carbon addition to wheat bran in koji flasks on phytase production by *A. niger*

Carbon Source	Phytase production as % of control*	Carbon Source	Phytase production as % of control*
0.5% Glucose	91.42	1.0% Glucose	92.08
0.5% Sucrose	83.5	1.0% Sucrose	81.31
0.5% Soluble starch	85.3	1.0% Soluble starch	78.9
0.5% Mannose	87.78	1.0% Mannnose	105
0.5% Lactose	83	1.0% Lactose	55
0.5% Maltose	68	1.0% Maltose	69
0.5% Sorbitol	57	1.0% Sorbitol	72

*control represents the total phytase activity produced from unsupplemented growth medium. (Richard Murphy, Internal Report, 2005, Alltech Bioscience Centre, Dunboyne, Co. Meath, Ireland).

Wheat bran represents the primary nitrogen source for the organism as it contains 18% protein (2.88% nitrogen). Additional nitrogen can be added to the fermentation medium as a supplement e.g., urea and soybean meal. Sumantha *et al.* (2006), working with a *R. microsporus* strain for protease production on a rice bran substrate, examined sixteen nitrogen sources at 1% (w/w), eight organic sources (beef extract, casein, corn steep liquor, corn steep solids, malt extract, peptone, tryptone, and yeast extract) and eight inorganic sources (NH_4HCO_3, $NH_4H_2PO_4$, $(NH_4)_2HPO_4$, NH_4NO_3, $NaNO_3$, KNO_3, $(NH_4)_2SO_4$, and NH_4Cl). Of the inorganic sources, only NH_4HCO_3 showed a slightly higher activity than the control and since the other inorganic nitrogen sources did not improve activity of the protease enzyme, this suggested that it was not the nitrogen present in the NH_4HCO_3 but rather it was the presence of the carbonate group that was responsible for the positive effect. Anupama and Ravindra (2001) reported that

carbonate, as a constituent of extraction buffer, resulted in increased protease recovery from rice bran fermented with *A. niger*. With the organic nitrogen supplements, although casein showed a slight effect, there was no large effect observed with any of the organic nitrogen supplements examined at this particular concentration (1% (w/w)).

These results were consistent with trials carried out in the production chambers in the plant over the past three years, as well as work carried out independently at Alltech's European Bioscience Centre in Ireland, looking at the use of different nitrogen supplements. A number of experiments were carried out and these included the use of peptone, cas amino acids, ammonium sulphate, ammonium phosphate, and urea at levels of 1% and 2%. Table 3.21 highlights the results. None of the nitrogen sources tested at these levels resulted in a significant and reproducible increase in phytase activity.

Table 3.21 Effect of nitrogen addition to wheat bran in koji flasks on phytase production by *A. niger*.

Nitrogen Source	Phytase production as % of control*	Nitrogen Source	Phytase production as % of control*
1.0% peptone	99.7	2.0% peptone	101
1.0% cas amino acids	96.5	2.0% cas amino acids	75
1.0% ammonium sulphate	99.5	2.0% ammonium sulphate	73.6
1.0% ammonium phosphate	111.8	2.0% ammonium phosphate	27
1.0% urea	30.9	2.0% urea	49.6

*control represents the total phytase activity produced from unsupplemented growth medium.

Similarly, past plant experimentation into the use of nitrogen supplementation for

protease production has not yielded any promising candidates for further detailed trials.

The type of nitrogen is important in terms of the possible effect on enzyme production and it has also been reported to have an effect on fungal morphology. Both *A. oryzae* and the fungus *Leptochloa chinensis* when cultured in liquid medium in shake flasks, have both been reported to exhibit a pattern varying between the filamentous form and a clumping form (Wan *et al.*, 2005). As mentioned in Methods Section 3.4.1, the presence of clumps during the liquid scale up process was used by the production plant as an indicator of potential problems during scale up. Based on past experience, flasks with fungal clumping were not good candidates for the scale up process and when used, consistently resulted in lower product yields. Consequently, flasks such as these were discarded and not used for scale up whenever this phenomenon was observed. It is speculated that it could be a nutritional deficiency causing this clumping problem.

It has been suggested that the ratio between carbon and nitrogen (C:N) is a crucial factor for a particular process to obtain a specified product. The ratios of C:N in the biomass in the medium are related to the yield and the biomass composition. A C:N ratio of sixteen is frequently reported to be suitable for fermentation processes. However, when dealing with the production of a product that is partially or totally non-associated with growth, the C:N ratio can vary.

3.6.1.2 Supplementation with gluten, glycerol, soya, and starch

Numerous reports in the literature have suggested that the addition of carbohydrate, protein or other nutritional substances to substrates such as wheat bran can be beneficial in terms of enhancing fungal growth and production of secondary metabolites. There is however much controversy in the literature regarding addition levels and effects (Krishna, 2005; Shankar and Mulimani, 2007; Singh and Satyanarayana, 2007). Nutrient supplementation appears to be very strain and process dependent.

Following examination of carbon and nitrogen addition, four supplementation substrates were investigated: glycerol, soya, starch and gluten. These were added at varying levels to the standard wheat bran.

Glycerol, a tri-alcohol, was added to the koji with the idea that since it is also a humectant, it might prevent moisture loss, enhance enzyme activity and stimulate the production of phytase. Gluten and soya are both inexpensive sources of protein and were added to the bran to see if the protein enhanced the production of the enzyme. Corn starch was chosen as a source of complex carbohydrates. Koji flask fermentations were analysed at minimum in duplicate and the majority in triplicate for phytase activity.

Table 3.22 Phytase activity (SPU/g) after 3, 4 and 5 days of fermentation at 30°C in koji flasks supplemented with glycerol, soya, starch and gluten at three additions.

Day	Control SPU/g	Glycerol 5%	Std Dev	Soya Meal 5%	Std Dev	Starch 5%	Std Dev	Maize Gluten 5%	Std Dev
3	456	349	156	365	31	264	57	314	50
4	899	634	145	760	89	672	48	603	33
5	1387	1180	12	1087	28	1343	29	1247	23

Day	Control SPU/g	Glycerol 10%	Std Dev	Soya Meal 10%	Std Dev	Starch 10%	Std Dev	Maize Gluten 10%	Std Dev
3	456	219	31	225	29	349	70	336	30
4	899	382	71	764	96	684	122	724	108
5	1387	222	3	1058	19	1040	29	1346	23

Day	Control SPU/g	Glycerol 15%	Std Dev	Soya Meal 15%	Std Dev	Starch 15%	Std Dev	Maize Gluten 15%	Std Dev
3	456	122	19	154	30	182	29	306	27
4	899	110	9	618	137	695	84	612	41
5	1387	222	4	1058	28	1040	18	1346	11

The addition of the supplements at the concentrations employed did not result in any increase in phytase production at the 5% level (Table 3.22). However, at the 10 and 15% level, especially with soya, starch and gluten, phytase enzyme level production was depressed.

When moisture levels were examined over the fermentation period (Table 3.23) it appeared that the glycerol addition did have a small positive effect on moisture levels, but since the addition did not increase the amount of phytase enzyme produced, glycerol was not further investigated.

Table 3.23 The % moisture after 3, 4 and 5 days of fermentation at 30°C in koji flasks supplemented with glycerol, soya, starch and gluten at three additions.

	Control	Level of Addition	Glycerol	Soya	Starch	Gluten
% Initial moisture	48.90	5%	51.60	45.28	52.36	49.17
		10%	50.72	48.70	45.10	49.60
		15%	53.90	50.54	49.38	49.90
% Moisture Day 3	49.13	5%	51.45	48.69	49.08	47.96
		10%	49.90	47.80	49.02	49.24
		15%	50.85	46.56	48.66	50.04
% Moisture Day 4	50.75	5%	50.92	51.36	49.42	49.64
		10%	48.93	47.73	49.60	50.82
		15%	52.07	48.61	50.13	50.22
% Moisture Day 5	52.53	5%	51.95	47.89	51.81	50.61
		10%	55.02	47.71	52.61	50.95
		15%	50.71	48.61	52.19	52.67

The pH was also monitored throughout the fermentations (Table 3.24) and the addition of the nutrients, not surprisingly, affected the pH to some degree, as growth was slowed in some of the flasks due to the higher addition of the four materials.

Table 3.24 The pH after 3, 4 and 5 days of fermentation at 30°C in koji flasks supplemented with glycerol, soya, starch and gluten at three additions.

	Control	Level of Addition	Glycerol	Soya	Starch	Gluten
Initial pH	6.36	5%	6.13	6.33	6.28	6.20
		10%	6.23	6.41	6.31	6.02
		15%	6.19	6.36	6.37	5.85
Day 3 pH	3.89	5%	3.69	3.99	3.82	4.07
		10%	4.49	4.36	4.07	3.95
		15%	5.95	4.65	4.33	3.90
Day 4 pH	3.99	5%	3.50	4.04	3.73	3.99
		10%	3.68	4.14	3.60	3.97
		15%	4.21	4.16	3.55	3.94
Day 5 pH	4.17	5%	3.54	4.03	3.80	4.15
		10%	3.38	4.09	3.61	4.17
		15%	4.06	4.24	3.38	4.25

Due to the disappointing results with the addition of these materials, no further work was carried out using these four materials as supplements.

3.6.1.3 Supplementation with yeast extract

Yeast extract is a valuable addition to microbial growth media and is routinely used as an additive for fermentation. A considerable market exists for such applications. This is due to the rich nature of the yeast intracellular content, which consists of high levels

of nitrogen, amino acids, vitamins, minerals and other nutrients that may be lacking in a fermentation medium. It is an ideal addition to fermentation media when one is concerned about the nutritional requirements of a strain, because of the many trace elements it contains. When biotechnologists want to work on improving fermentation, they will often investigate the effects of yeast extract before trying more specific supplements. This is particularly important when strains are used that have undergone mutation treatment to enhance enzyme production ability, as the mutation process is not specific and there are often hidden mutations that can result in growth problems when the strain is subjected to stress. This microbial nutrient 'kitchen sink' can vary from supplier to supplier as it is of course produced from a microbe itself. Depending on how the yeast fermentation is managed and the properties of the yeast strain itself, the extract produced from that particular yeast may vary significantly. The yeast extract used for this study was produced from primary baker's yeast and the manufacturer's specification sheet is appended (Appendix D).

Fermentations were carried out with yeast extract supplementation of the medium. Yeast extract addition to wheat bran was at a level of 1.67% (8kg/480 kg batch) in production chambers over a one month period. The control was a standard fermentation carried out in the same chambers and under the same conditions. Location in the chamber was varied. Table 3.25 illustrates the effect of yeast extract on production scale fermentations when examined with 67 batches, and in all six chambers, to ensure that the results were consistent and not a batch to batch or chamber variation effect.

Table 3.25 Effect of yeast extract supplementation on phytase production.

Liquid Inoculum	Control SSF (SPU/g)	SSF With Yeast Extract Added (SPU/g)	Difference as SPU/g	Difference as a %
SF 1261	414	542	128	30.9%
SF 1268	258	316	58	22.4%
SF 1269	234	343	109	46.5%
SF 1270	377	462	85	22.5%
SF 1271	439	508	69	15.8%
SF 1272	299	411	112	37.4%
SF 1273	282	396	114	40.4%
Totals	329	425	96	30.8%

An average improvement of 30.8% was seen during the experiments where yeast extract had been added to the bran. This result was significant and very promising for future work investigating the exact material in the yeast extract causing this improvement to take place.

Subsequent work will focus on other levels of yeast extract addition, further investigation into the content of this yeast extract and supplementation of materials identified in the yeast extract individually and in combinations to narrow down the material or materials that are responsible for this improvement. The mineral composition of the raw materials used is given in Table 3.26.

Table 3.26 Summary of mineral composition.

	Wheat bran	S4 Inoculation Media	Yeast Extract	Water
Calcium	0.85 - 0.11%	0.71%	0.08%	132 ppm
Copper	13 – 18 ppm	< 1 ppm	<1 ppm	0.048 ppm
Iron	123 -161 ppm	< 5 ppm	24 ppm	2.7 ppm
Magnesium	0.40 - 0.55%	<0.01%	0.17%	0.024%
Manganese	145 -163 ppm	55 ppm	1 ppm	0.15 ppm
Phosphorous	1.01 -1.28%	0.84%	1.83%	1.1 ppm
Potassium	1.23 -1.36%	1.48%	3.36%	Not analysed
Sodium	< 0.01 -.02%	0.47%	2.61%	50 ppm
Sulphur	0.18 -0.20%	0.01%	1.25%	5.54 ppm*
Zinc	85 -103 ppm	55 ppm	22 ppm	3 ppm

* as sulphate

Another consideration is the bioavailability of the trace metal in the substrate. Analysis may indicate that a trace metal is there at a concentration that does not appear too low, but the metal may not be available to the organism (Kreder, 1999). From this information it was suggested that opportunities exist for trace mineral supplementation to improve enzyme production.

3.6.2 Effect of fermentation parameters

Other than supplementation of the medium, modification of fermentation parameters and controls is one of the most effective ways of improving fermentation systems. It is also probably the most important methodology, since lack of control over the fermentation will always end in poor results since the optima for a fermentation in basic parameters must be found through experimentation or trial and error.

3.6.2.1 pH (effect of initial ph on phytase and protease activity)

Using the standard koji flask experimental procedure (Methods Section 2.8.1) an experiment was conducted to determine the effect of changing one variable. The initial pH of the bran substrate was lowered to examine the effect on the level of phytase enzyme produced and on the weight of bran material lost.

The pH of the bran was adjusted using HCl to yield 200 ml flasks with a starting pHs of 6.58, 5.40, 5.27, 5.04, 4.89 and 4.66. The change in pH over time and the level of phytase enzyme present in the flask were determined after 3 and 4 days incubation at 30°C.

In all cases where the starting pH of 6.58 was lowered with HCl addition, a reduction in phytase enzyme production at both day 3 and day 4 was observed. The lower the starting pH, the greater the effect on both phytase production and lost weight from the tray.

Tray lost weight is considered to be an indirect indicator of the amount of biomass that has been consumed by the organism (i.e., has been lost as heat, water and/or CO_2) and was calculated by weighing the flask after inoculation and after 3 and 4 days fermentation. The greatest weight loss was seen in the flask where the pH had not been lowered and this flask also showed the highest phytase enzyme activity after both day 3 and day 4 inoculation (Table 3.27).

Table 3.27 Effect of initial koji bran pH in 200 mL flasks on final pH, phytase levels and tray lost weight.

Day 0 Initial pH	Day 3 pH	Day 4 pH	Day 3 Phytase activity	Day 4 Phytase activity	Day 3 Tray Lost Weight (%)	Day 4 Tray Lost Weight (%)
6.58	3.90	3.96	378	613	23	32
5.40	3.91	3.72	216	475	22	30
5.27	5.30	3.38	110	313	19	26
5.04	5.33	4.64	BDL	4	19	22
4.89	5.36	5.15	BDL	BDL	18	23
4.66	5.23	4.80	BDL	BDL	16	21

All values on table are the average form a minimum of 3 samples

BDL = Below Detectable Limits

Numerous authors have observed a good correlation between growth and organic acid production. Organic acid production can be estimated using pH measurements or HPLC analysis of the substrates. Soccol (1992) demonstrated a close correlation, using a strain of Rhizopus between fungal protein (biomass) and organic acid production (citric, fumaric, lactic or acetic). Production of organic acids results in the pH being lowered, but there can also be assimilation of organic acids from the medium by the fungus. This can lead to an increase in pH (Krishna, 2005). The greatest decrease in pH was seen in the flasks with a starting pH above 5.0 (Table 3.27).

Flasks with a starting pH below 5 showed the lowest levels of phytase enzyme activity although they still showed a weight loss of ~22% indicating that there was biomass consumed by the fungus.

Filamentous fungi can grow over a broad pH range (pH 2 - 9) with an optimal range around pH 3.8-6.0 (Walker, 1998a). By choosing a lower pH, one can exploit the ability of the fungus to grow at the low pH where bacteria are normally inhibited, and use this to prevent, or at least minimise, bacterial contamination. Thus, a low pH is very important in terms of preventing contamination on the koji trays. However, it must be remembered that there are some bacteria that can grow at a very low pH. Although it is possible to adjust the starting pH by acid or base additions to the wheat bran, pH control during the fermentation is extremely difficult. In a submerged fermentation in a tank, pH control is easily accomplished, but in a SSF with the numerous individual trays, and each tray covered with a lid, pH adjustment is not physically or economically feasible on a production scale. Attempts have been made to overcome the problem of pH variability by selecting components with a buffering capacity. The buffering capacity of the bran medium used is discussed in Methods Section 2.4.4 of this thesis.

For optimisation in the plant, it is necessary to balance a lower starting pH for the koji, which will reduce bacterial contamination over the growth period versus utilising a higher pH, which will yield higher phytase activity, but be more susceptible to contamination.

3.6.2.2 Temperature and phytase

Figure 2.32 in the Methods Section of the thesis is an illustration of a typical temperature profile seen in a fermentation chamber. Warming the chamber required the use of warm water, but only for a short period of time as the heat of fermentation quickly caused the temperature to rise in the chamber and cooling quickly became the priority to ensure that the chamber did not reach temperatures that would inhibit enzyme production by the fungus.

Constant temperature was examined as a single variable experiment with *A. niger* in koji flasks. When the flasks were held at 30°C, the fermentation for phytase enzyme production showed considerable advantage over the other fermentation temperatures examined (Figure 3.27). A sharp drop was noted at 35°C, and the reduction in phytase activity was most pronounced at 40°C. The results of the koji flask trial suggested that the temperature optimum was probably close to 30°C for this particular strain, under these experimental conditions, and that higher temperatures would result in a reduction of enzyme produced.

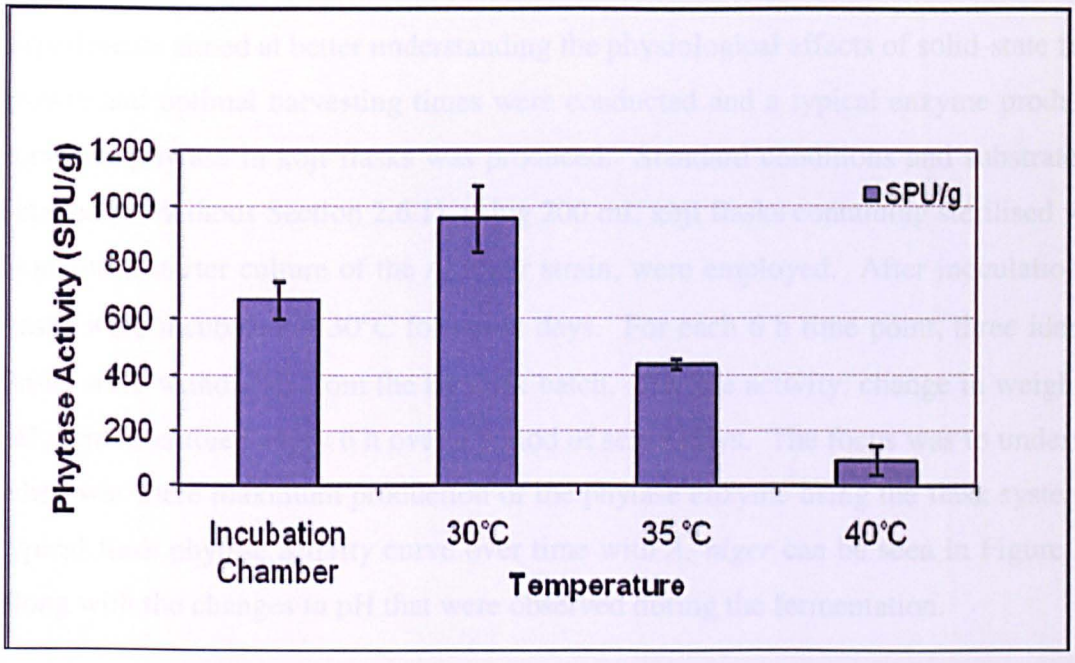


Figure 3.27 Effect of a constant growth temperature for 96 h on phytase activity in koji flasks. Duplicate flasks were tested and error bars indicate standard deviation.

These results agreed with the studies carried out by Carter *et al.* (2004), who examined the growth of a strain of *A. niger* used for commercial phytase production at 23°C, 30°C and 35°C, on both wheat bran and soybean meal, and found that 30°C yielded maximum phytase production. Growth temperature is a very critical parameter and varies with the particular organism, but at higher temperature due to the production of metabolic heat, the fermenting substrate temperature can quickly rise past the ability of the system to remove the heat and result in inhibition of microbial growth and enzyme formation. Thus, the temperature choice for the fermentation chamber must be a balance between

the optimal temperature for the organism to grow and secrete enzymes, balanced against what the system can offer in terms of heat removal capability. With the koji flasks, maintaining a constant temperature in a laboratory incubator is easily achieved, but maintaining a constant temperature in every part of a large fermentation chamber loaded with over 1000 trays is extremely difficult.

3.6.2.3 Time course of phytase enzyme production by A. niger in koji flasks

Experiments aimed at better understanding the physiological effects of solid-state fungal growth and optimal harvesting times were conducted and a typical enzyme production curve for phytase in koji flasks was produced. Standard conditions and substrates (as detailed in Methods Section 2.6.1), using 200 mL koji flasks containing sterilised wheat bran and a starter culture of the *A. niger* strain, were employed. After inoculation, the flasks were incubated at 30°C for seven days. For each 6 h time point, three identical flasks were withdrawn from the 84 flask batch. Phytase activity, change in weight and pH were monitored every 6 h over a period of seven days. The focus was to understand when was there maximum production of the phytase enzyme using the flask system. A typical flask phytase activity curve over time with *A. niger* can be seen in Figure 3.28, along with the changes in pH that were observed during the fermentation.

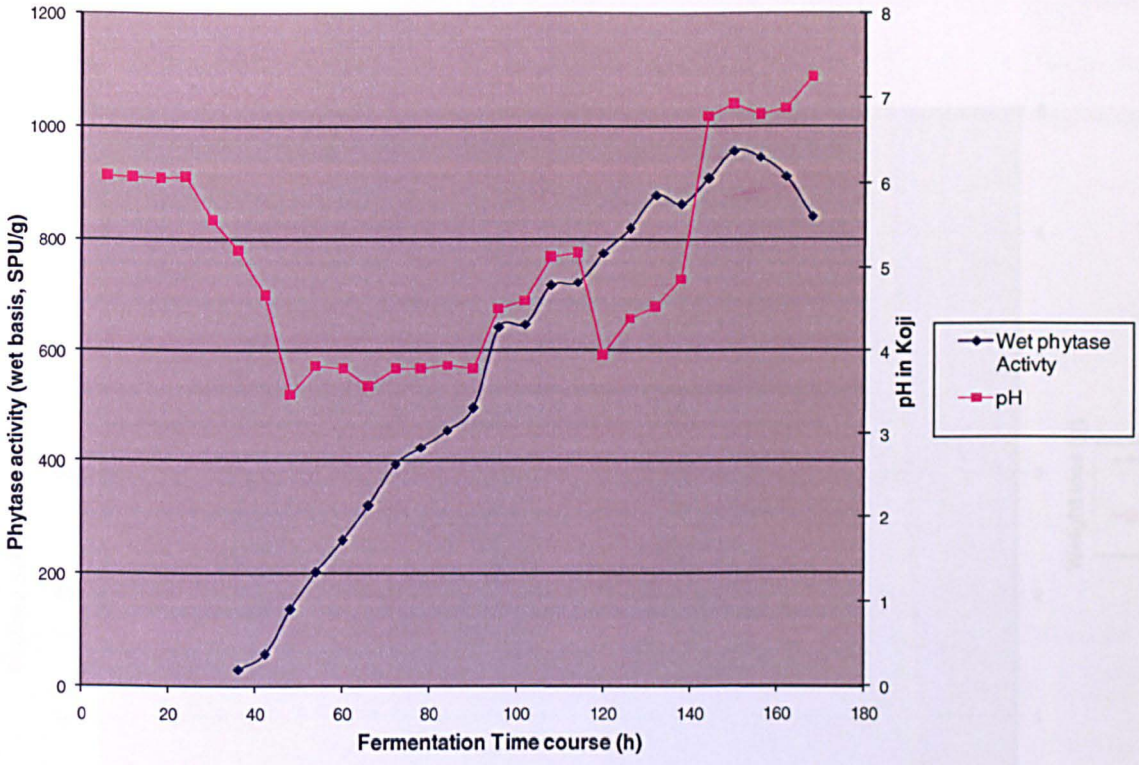


Figure 3.28 Typical phytase enzyme production curve, and change in pH in koji flasks inoculated with *A. niger* and incubated at 30°C.

Maximum enzyme levels were reached at six days and then the activity decreased. The phytase activity productivity was calculated to be 8.3 SPU/g-h. The pH was observed to drop significantly in the first two days, from pH 6.09 to pH 3.47. Then the pH remained steady until day four, when it rose again to finish higher than the initial measurement. The bran has the ability to buffer changes in pH to a degree as seen in Methods Section 2.4.4, where the buffering capacity of bran was assayed. During the fermentation, the pH fell as the organism produced organic acids and carbon dioxide and then rose again as ammonia was produced later in the fermentation (as discussed in the Introduction section 1.2.4.4).

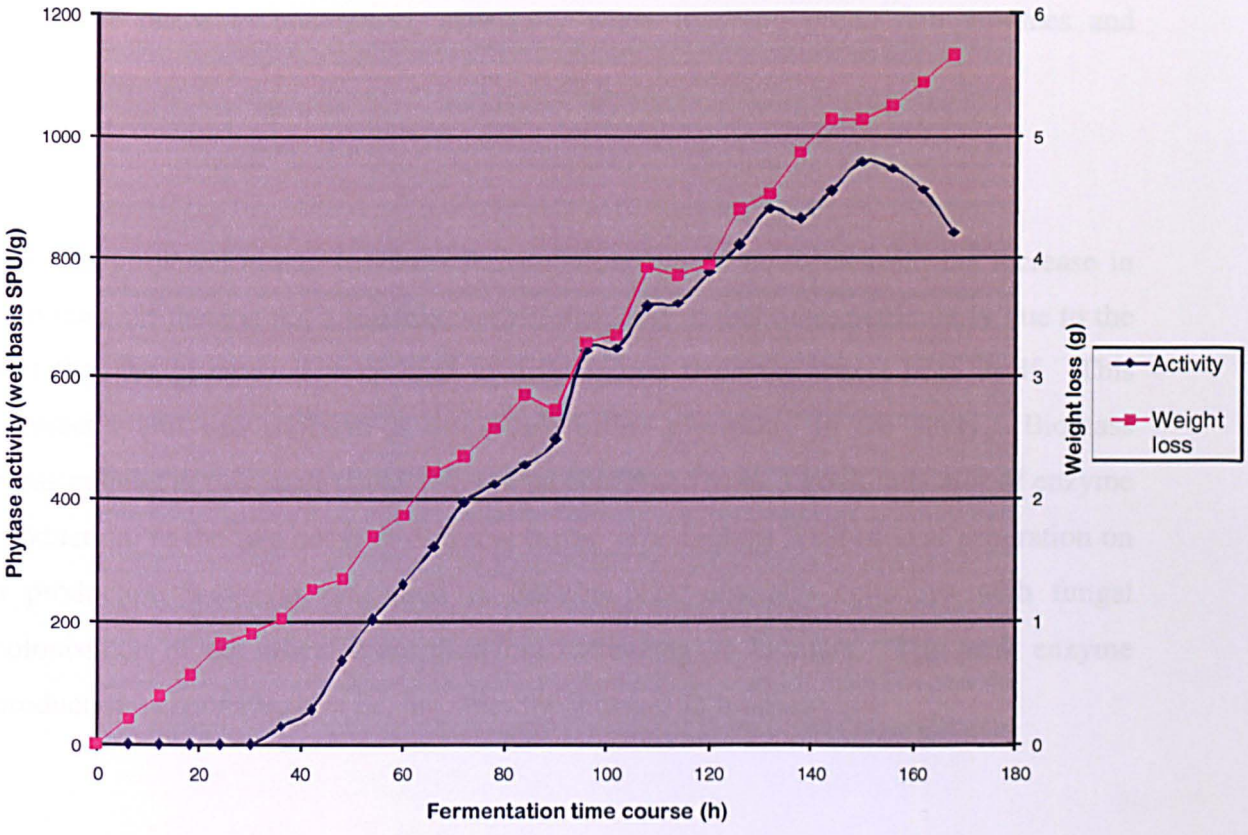


Figure 3.29 Typical phytase enzyme production curve (calculated on a wet basis) and weight loss in koji flasks inoculated with *A. niger* and incubated at 30 °C.

Over the seven day fermentation there was a consistent loss of weight from the flask as the wheat bran was consumed by the fungus (Figure 3.29). The initial weight of the koji in the flask decreased during the seven days of the fermentation and at day seven was at about 60% of the initial weight. The fungus utilises the wheat bran during the fermentation in order to multiply and produce more biomass, as well as producing enzymes. Biomass can be produced at the price of enzyme production. On a production scale, overactive mycelial growth has sometimes been seen as an indicator of an unhealthy fermentation, where too much biomass is produced instead of enzymes. This is often attributed to moisture levels being too high (Y. Yamamoto, Shin Nihon Chemical Company, Anjo, Japan, personal communication). Monitoring loss in weight

gives us an idea of the progress of the fermentation. On a plant scale, this is a very difficult method to implement due to the handling that would be required, but it is an important factor to understand, especially when planning production volumes and scheduling.

Another proposed option to assess fermentations would be to measure the increase in biomass, but there is not a reliable method available at this time, particularly due to the fact that the biomass is very hard to differentiate from the wheat bran itself. This parameter and this problem is discussed further elsewhere in the study. Biomass measurement at this stage could also not be confirmed to be a good indicator of enzyme production, as the two occur at different times. The highest level of heat generation on a production scale (as discussed in Section 3.2) generally coincides with fungal colonisation of the wheat bran, therefore increasing its biomass. The peak enzyme production occurs after this i.e, just after the increase in biomass.

Moisture of the koji increased slightly over time from 52% moisture at inoculation time to a maximum of 60% at day 5 (Fig. 3.30).

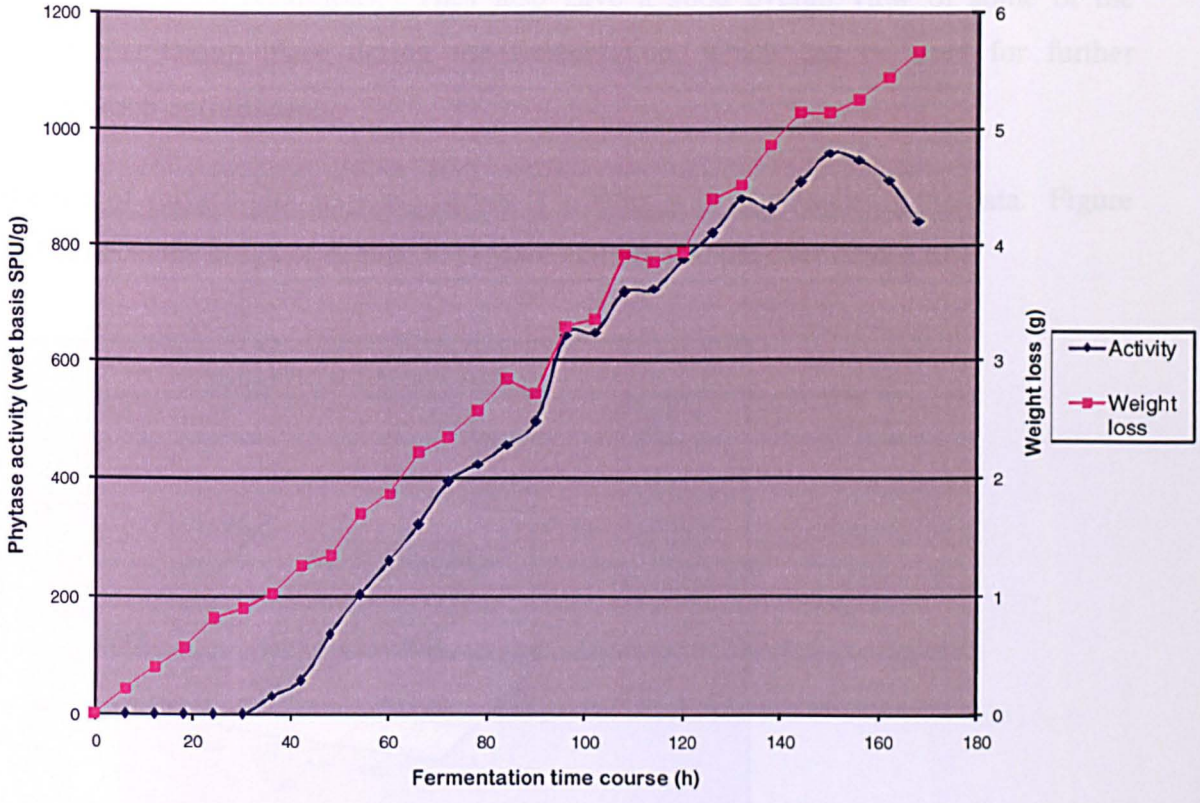


Figure 3.30 Typical phytase enzyme production curve (calculated on a wet basis) and changes in moisture in koji flasks inoculated with *A. niger* and incubated at 30°C.

Although moisture has traditionally been measured in these types of fermentation, as discussed later in the study, moisture content of the solid substrate is not a precise measurement for predicting what is available to the fungal culture (Raimbault, 1998; Oriol *et al.,* 1998), and is more of an indicator of whether the environmental conditions in the incubator are under control. An increase in the moisture in the koji flasks on day 5 is the result of respiration of the organism, creating condensation in the flask, that forms and falls back down into the substrate.

These findings were interesting as they varied from the production scale observations and higher activities were produced than normally seen on a plant scale, suggesting that the ideal parameters may be more similar to those used in the laboratory scale tests than under plant scale conditions. They also gave a good overall view of some of the phenomena taking place during the fermentation, which can be used for further fermentation optimisation.

The use of surface plot response curves is a better way of visualising the data. Figure 3.31 reflects the effect of change in phytase activity and pH over days 4 to 7.

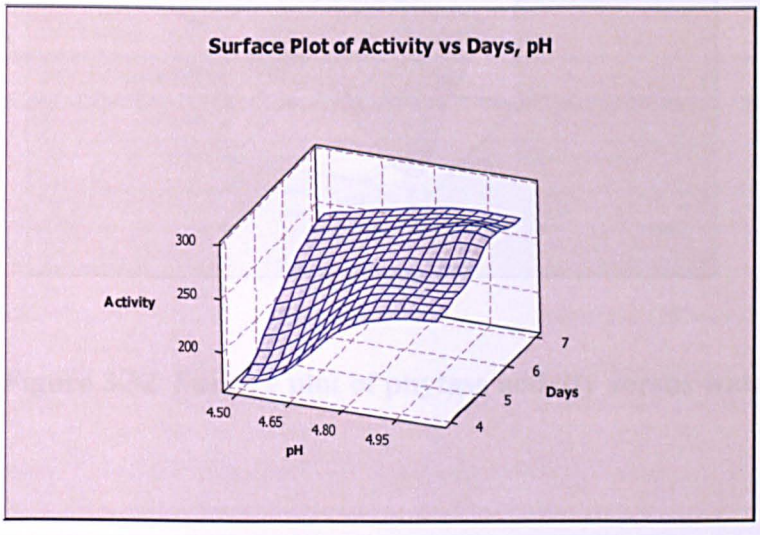


Figure 3.31 Surface plot of phytase activity versus days and pH.

Figures 3.32 and 3.33 reflect the effect of change in phytase activity comparing the plots of water activity (A_w) and moisture.

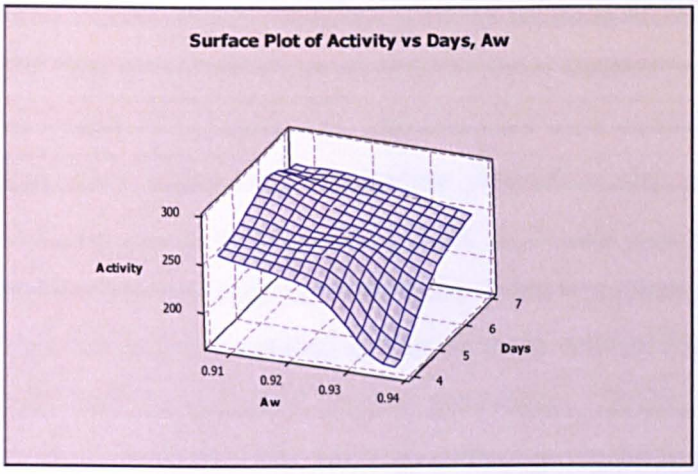


Figure 3.32 Surface plot of phytase activity versus water activity (A_w) and days.

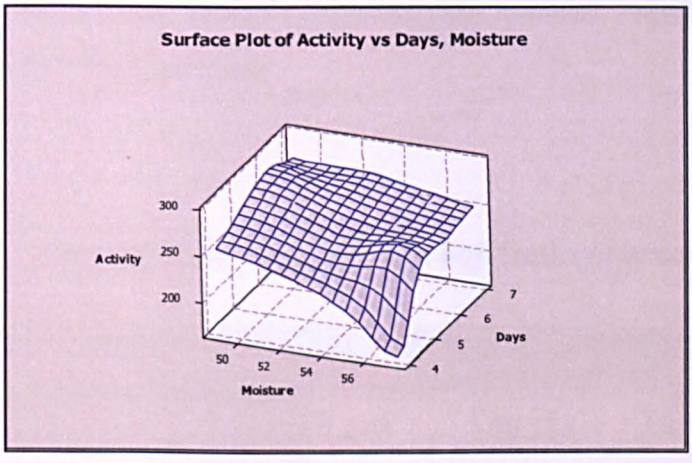


Figure 3.33 Surface plot of phytase activity versus % moisture and days.

Comparing the surface plots of the water activity and the % moisture one can see similar patterns and yet some differences. Again, this is as what would be expected since moisture is a crude measurement, while water activity is a more useful measure in

terms of what water is available to the organism.

3.6.2.4 Time course for protease production by A. oryzae in koji flasks.

Protease activity with the strain *A. oryzae* was examined in koji flasks over a 5 day period (Table 3.28). Methods were as outlined in Methods Sections 2.8.1 and 2.11.3 (koji flask preparation and protease analysis method) with samples analysed at 1, 2, 3, 4 and 5 days of fermentation. All analyses were carried out in triplicate. Although the strain of *A. niger* used for phytase production also produces protease, its production levels are much lower. The fungus *A. oryzae* was utilised for fermentations where protease was the main enzyme focus. This is a much more aggressive culture in terms of growth rate and exhibits higher protease activity than what is seen with the *A. niger* strain used for industrial phytase production. Quality control data over time indicates that an average of 1,500 (HUT/g) units is produced from the *A. niger* strain (considered a side activity for marketing purposes), while an average of 40,000 (HUT/g) units is produced from the *A. oryzae* strain. The *A. oryzae* strain grows more quickly on the koji and when the goal of the fermentation is strictly protease enzyme, the koji can be harvested in as little as 72 h. With the more aggressive growth by the fungus and the consequently shorter incubation time required, contamination is much less of an issue in these fermentations.

Table 3.28 Protease activity in koji flasks over a 5 day period.

Time Period	Day 1	Day 2	Day 3	Day 4	Day 5
Average Activity * (HUT/g)	1,643	20,536	43,174	12,487	9,119
Standard Deviation	523	996	4,991	1,861	2,205
Spores observed	NO	NO	NO	Yes	Yes

*analyses carried out in triplicate

The highest protease activity was observed at day 3 during incubation in koji flasks and strain did not sporulate in koji flasks until day 4 of incubation. These spores can cover product and increase in numbers very rapidly. As mentioned earlier in this document, sporulation by the fungus is to be avoided due to both health and safety issues regarding the presence of spores in the air. In addition, protease activity declines after day three. It is speculated that perhaps the protease is deactivated or that the drop is due to the high growth in the mycelium and re-absorption of this protein for maintenance of the fungal mycelium.

Further work on reducing or eliminating sporulation would need to be carried out in order to use this strain commercially. However, the protease activities were at a high enough level where they would be of interest.

3.7 Multiple Variable/ Quadratic Optimisation Experiments

3.7.1 Fermentation optimisation – An overview of techniques

In the biotechnology industry, fermentations are typically designed for the production of a specific chemical product, but there may be two or more desirable products from a fermentation. For the production of a single product, the goal of optimisation is to produce this product a least cost. Normally, the two main factors determining the cost of the product are volumetric productivity of the microorganism and the yield of product based upon the major raw material. Occasionally, the costs of other raw materials, utilities, etc., are major factors in the minimisation of the overall cost of production. When there are a number of products desired from a fermentation, this optimisation becomes even more complex.

There are many independent variables that influence a fermentation and these can be modified to improve it such as the temperature, the pH of the fermentation, the strain of

the microorganism, the myriad medium components, aeration, mixing, etc. Statistical techniques permit the analysis of data taken around a set of variables to determine which have an effect upon the production of the product in this range of variables. These statistical analyses provide important insights into the factors and potential mechanisms involved in the fermentation (see previous sections). They also suggest how various independent variables, such as pH, can be modified to improve the fermentation.

The statistical analyses only provide information in the region of parameters (independent variables) used in developing the data. Since values of the independent variables that will give improved production of the desired product probably lie outside the region of parameters used in the statistical analysis, other techniques need to be employed that can give improved production of the desired product. Methods used to find the values of parameters are called search techniques or optimisation techniques.

The discussion, which follows, is useful for laboratory optimisation of fermentations wherein the random effects of fermentation can be minimised. It should be noted, that typically fermentations involve too many variables to achieve what would be called an optimal fermentation. Therefore, fermentation optimisation is in fact continual fermentation improvement.

Search and Optimisation Techniques

There have been several journals devoted to optimisation techniques and many books written on this subject such as Foundations of Optimisation by Wilde and Beightler (1967) and Optimisation: Theory and Practice by Beveridge *et al.* (1970). Probably the most used optimisation technique is the Linear Programming Simplex Method initially developed by Dantzig (1963).

These optimisation techniques are usually described as mathematical methods for finding the optimal values of an objective function. The objective function is the relationship of the dependent variable on the independent variables. Sometimes this objective function can be expressed analytically. As an example, the final concentration of a chemical formed by a chemical reaction can be mathematically described by equations that quantify the relationship of the final concentration with the temperature, the time of reaction, and the initial concentrations of the reactants. The objective function in this case would be the analytical and various analytical optimisation (as well as numerical) techniques that could be used with this analytical objective function.

In the case of fermentation optimisation, typically no mathematical function exists which describes the dependent variable (e.g., final enzyme concentration) as a function of the independent variables such as starting pH, temperature, and medium components. The objective function is determined by experimentation i.e., experiment or series of experiments are performed and the dependency of the dependent variable on the independent variable is found.

The techniques applicable for fermentation optimisation are then limited to those which can use experimentation to define the objective function. There are many optimisation techniques that can be used with experimentally determined objective functions.

The selection of optimisation techniques is determined by the characteristics of the fermentation improvement process. Typically, no analytical formula exists that describes the dependent variables such as yield, concentration, or productivity of the fermentation as a function of the parameters (independent variables). The relationship of the dependent variables to the independent variables is determined by experimentation. Therefore, no analytical optimisation can be used.

There are various numerical techniques that can be used for the optimisation of

dependent variables that are experimentally related to the independent variables i.e. the objective function is determined experimentally. Some of these search techniques involve obtaining one value of the objective function for each move of the independent variables toward the optimum e.g. parallel tangents and the simplicial method. These methods are not well suited to fermentation optimisation because each fermentation experiment may require days or even weeks. Therefore, the progress of the optimisation would be very slow.

Other search methods such as the sequential single dimensional search (searching for the optimal value for one independent variable at a time) are also time consuming and can lead to incorrect assumptions. Similar techniques such as the pattern search are better, but also time consuming.

The best search techniques for fermentation optimisation are techniques that permit the performance of many experiments at one time. The extrapolation from the results of these experiments to new conditions can give improved values for the dependent variable. Among the methods that utilise many experiments to extrapolate to new conditions for the optimisation of the dependent variable are gradient techniques, quadratic fit techniques, genetic algorithm techniques, and neural network techniques. These techniques by themselves, and in combinations, have been the main procedures used in fermentation improvement. Which technique is chosen is somewhat arbitrary and there is little ability to determine *a priori* which method will best work. The quadratic method, with some minor variations, will be discussed in detail. This discussion will give some idea as to how fermentation optimisation is utilised.

The gradient technique basically involves selecting a base point (some values for the independent variables e.g. values for pH, temperature, and two medium components). Small variations in each of the independent variables (four independent variables in the example) are then made about this base point. The value of the dependent variable (e.g. enzyme activity) is then simultaneously experimentally measured at the base point and

each of variations. From this set of simultaneous experiments, the gradient in the dependent variable (e.g. enzyme activity) as a function of the independent variables (e.g. pH, temperature, and two medium components) is calculated. This gradient suggests a direction to move the independent variables to improve the dependent variable. The independent variables are moved a predetermined distance in the direction of the gradient and the process of determining the gradient is repeated with another move in the independent variables. This method works well if the experimental measurements of the dependent variable (e.g. enzyme activity) contain little random error. However, for many fermentations, the random errors in the measurements are considerable and preclude the calculation of an accurate gradient thus reducing the utility of the gradient method.

Another method used in the fermentation optimisation is the genetic algorithm. This method assumes that each of the independent variables is part of a genetic code and can be changed by random variations. For each of the independent variables, a variation in that variable, which will cause a significant change in the dependent variable, is determined. Some base point (particular values for the independent variables) is selected and random variations in these independent variables are made as if a mutation in the variable had occurred. Simultaneous experiments are performed at the base point and the points of mutation. From these measurements, one of the points (specific values of independent variables) will yield a maximum value of the dependent variable. This point is then selected for the next base point for the genetic algorithm. The procedure is continued until a maximum is found for the variables selected.

Some other techniques such as neural networks, stochastic optimisation, etc., can be used for fermentation optimisation. One method for improvement of fermentation is to utilise quadratic approximations for the objective function (called response surfaces in statistics) and use the optimum values found from this approximation to generate the next point for optimisation. A variation in this procedure is as follows.

Quadratic Optimisation

This procedure again is similar to the gradient and genetic algorithm methods in that experimental measurements are made around a base point and the results of these experiments are used to project to the next base point. The method for the selection of the next base point is to approximate the data with a quadratic function, which has an optimal value somewhere within a restricted space around the base point. To explain the procedure, the optimisation in one dimension will be explained and then the extrapolation to the multidimensional case will be given.

In one dimension, the dependent variable, g (e.g. enzyme activity produced) can be expressed as a function of the independent variable, x (fermentation pH) by a quadratic formula as shown below.

$$g = a + b*x - c*x^2 \qquad (4)$$

If three or more measurements of g are made for three or more different values of x near an initial guess of the best value of x, it is possible to estimate the parameters a, b, and c using least squares minimisation. If the value of c is positive (c can be forced positive in the least squares minimisation), then this formula can be used to estimate a value of x for which g is a maximum. At the maximum, the derivative of g with respect to x is zero. Using this criterion, the x value for the maximum in quadratic fit function for g is given by the following formula.

$$x_m = b/(2c) \qquad (5)$$

Sometimes, this new optimal value of x is outside any range of feasibility and the range on x must be restricted. If this is the case, then the optimisation can be described as the

minimisation of the least squares error for experimental measurements and the prediction subject to the constraints on the optimal variable and the parameters in the quadratic approximation.

For the one-dimensional case, suppose that we have a base point at x_o and we make measurements at several points, x_i, where i is greater than 3.

Then we would have the following data points.

Independent variable (e.g., pH)	Dependent variable (e.g., enzyme activity)
x_o	g_o
x_1	g_1
x_2	g_2
x_3	g_3
x_4	g_4

The sum of the squared errors between the experimentally measured values and the predicted values is formed.

$$Z = \text{sum}(g_i - a + b*x_i - c*x_i^2)^2 \qquad (6)$$

Where this sum includes all the experimental data taken (in the example i would go from 0 to 4). The optimal x is determined by the following equation as above.

$$x_m = b/(2c)$$

This value of Z is then minimised subject to the following constraints.

$$C > 0$$

$$x_{min} < x_m < x_{max}$$

This new value of x_m can be used again as a starting point to measure values of g near this x value and the process can be continued. Sometimes adjustments are needed because the optimisation gives a lower value at the new point.

This process can be generalised into more than one independent variable (say n variables) as follows.

$$G = A + B*X - X*C*X \qquad (7)$$

In this formula B and X are vectors of dimension n and C is an nxn triangular positive definite matrix. Again, the values of A, B, and C can be estimated from measured values of G at different values of X. In this case, the optimal value for X will be given by the following formula.

$$X_m = C^{-1}B/2 \qquad (8)$$

The squared error can be minimised for the difference between the experimental data and the prediction to yield the parameters, A, B, and C. In this case, C will be a diagonal matrix.

The constraints on the minimisation of the least squares are as follows.

C is positive definite

$X_{min} < X_m < X_{max}$

The minimum number of measurement points depends upon the number of independent variables used in the optimisation. The minimum number of experiments is listed below. For n = 1, the minimum number is three as discussed above.

n	Minimum Number of Experiments
1	3
2	6
3	10
4	15

The theory requires a minimum number of experiments to be able to calculate the constants in the quadratic formula, which has been fitted to the data with least squares. However, there is typically error in the experimental data and that requires more measurements.

In order to understand the magnitude of the random error in the experiments, it is typically useful to perform all experiments in duplicate (or triplicate). Then the minimum number of experiments is at least double that listed above. Thus, with three variables, thirty to forty experiments are performed simultaneously.

3.7.2 Application of the quadratic fitting technique

Data was obtained for phytase enzyme activity in experimental trays in the SSF. The independent variables are the duration of the fermentations in days, the total zinc concentration, and the water content of the koji after inoculation. All trays were inoculated with the same culture. However, some parameters of the trays such as temperature and time of inoculation showed some random variation and therefore some errors are expected in the data. Because six tray fillings were used with this seed fermentation, six different trays were filled. The enzyme activity is the average of six trays. For these experiments, 108 trays were analysed. The data is shown in Table 3.29.

Table 3.29 Summary of experimental parameters.

Phytase Enzyme (SPU/g)	Days	Zinc (ppm)	Water (%)
296	4	40	51
221	4	40	54
214	4	40	54
260	4	109	48
192	4	109	52
174	4	109	55
204	4	178	48
150	4	178	50
144	4	178	54
337	5	40	51
233	5	40	54
230	5	40	54
359	5	109	48
236	5	109	52
299	5	109	55
267	5	178	48
261	5	178	50
214	5	178	54

The formula used to fit the data is as follows.

$$EA = a + b_1*x_1+b_2*x_2+b_3*x_3+c_{11}* x_1* x_1+c_{12}* x_1* x_2+c_{13}* x_1* x_3+c_{22}* x_2* x_2+c_{23}* x_2* x_3+C_{33}* x_3* x_3 \quad (9)$$

EA = enzyme activity

x_1 = days of fermentation

x_2 = total zinc concentration in ppm

x_3 = percent water in koji

This equation has ten unknown constants (described above for three independent variables) and these ten constants are determined by minimising the square of the difference between the experimental value and the predicted value. The constraints on the variables are as follows.

$$3 < x_1 < 6$$

$$20 < x_2 < 400$$

$$40 < x_3 < 60$$

This procedure was implemented on an Excel spreadsheet using the solver routine. Surprisingly, the solver routine found several minima when in theory there should be only one minimum. Sometimes multiple minima are found when several optimum points exist in a fermentation system. The sum of the squared error differed greatly from one minimum to another. The ten constants that result from the minimum squared error calculation are given below.

$$A = \begin{matrix} -23597.5336 \\ \\ 197.537971 \end{matrix}$$

$$B = \begin{matrix} 3.42148194 \\ \\ 914.032415 \end{matrix}$$

$$\begin{matrix} 16.4614976 & 0 & 0 \end{matrix}$$

$$C = \begin{matrix} -0.40382197 & 0.01033418 & 0 \\ 0.55965318 & 0.06779962 & 8.91371428 \end{matrix}$$

The inverse of the C matrix is given below.

$$C^{-1} = \begin{matrix} 0.06074781 & 0 & 0 \\ 2.37380203 & 96.7662463 & 0 \\ -0.02186974 & -0.73602484 & 0.11218668 \end{matrix}$$

From the calculation, the next point predicted to have a higher enzyme activity would be the following.

x_1 = 6 days of fermentation

x_2 = 400 ppm total zinc concentration

x_3 = 47.8% water in koji

Thus, the optimisation procedure recommends moving to the maximum number of days within the constraint, the highest zinc concentration within the constraint and an intermediate water content. It is still not understood why the Solver routine from Excel will not immediately find the true minimum of the sum of squared errors. When this difficulty is overcome, the procedure will be more robust. Once the optimisation procedure suggests a direction there is also the consideration of what can practically be applied in the plant and decisions can be made on where to run the next set of experiments or implement procedure into the production scale.

3.7.3 Koji flasks and optimisation of parameters

In order to carry out the multiple variable experiments, which include large numbers of samples, in a feasible way in terms of inoculation and sampling and control of the environmental conditions, koji flask experiments were employed even though it was clear that the micro-environment and the macro-environment were different and that conclusions on a small scale might not necessarily scale up in the same manner. Factors that had been highlighted as important, and amenable to manipulation, included pH, temperature and fermentation time. Mineral levels were of interest based on the positive effect when using yeast extract as a supplement, since yeast extract is known to contain high levels of zinc (Walker, 1998b). Koji flask fermentations were conducted in order to find some indication of where this optimum might lie.

Three trials were conducted and fermentations were set up to examine the effect of four variables (zinc, pH, moisture level, and time) following the standard procedures described in Methods Section 2.13 for phytase fermentations. All flask experiments were carried out in duplicate.

In Trial 1, zinc addition was 0, 1.5 and 3 ppm, pH was 4.5, 5.5, 6.5 and initial moisture was 46%, 50% and 54%. Samples were analysed after fermentation for four days and five days at 30° C. The experiment entailed the use of 108 koji flasks.

In Trial 2, zinc addition was 0, 0.3 and 0.6 ppm, pH was 4.5, 5.5, 6.5 and initial moisture was 46%, 50% and 54%. Samples were analysed after fermentation for four days and five days at 30° C. The experiment entailed the use of 108 koji flasks.

In Trial 3, zinc addition was 0, 2 and 4 ppm, pH was 4.5, 5.5, 6.5 and initial moisture

was 46%, 50% and 54%. Samples were analysed after fermentation for four days and five days at 30°C. Each experiment entailed the use of 108 koji flasks.

In koji flask Trial 1, 2 and 3, zinc had no significant effect or interaction at the concentrations added in the koji flask environment.

However, a relationship between pH and moisture could be seen in Trial 3. Contour plot Figure 3.34 and contour plot Figure 3.35 from Trial 3 illustrate the relationship between pH and moisture and show a significant linear and quadratic effect in both day 4 and day 5 activities. Day 5 shows an optimum pH level between 6.5 and 7.0 and that increasing moisture at that pH level leads to higher phytase activities (Fig. 3.35).

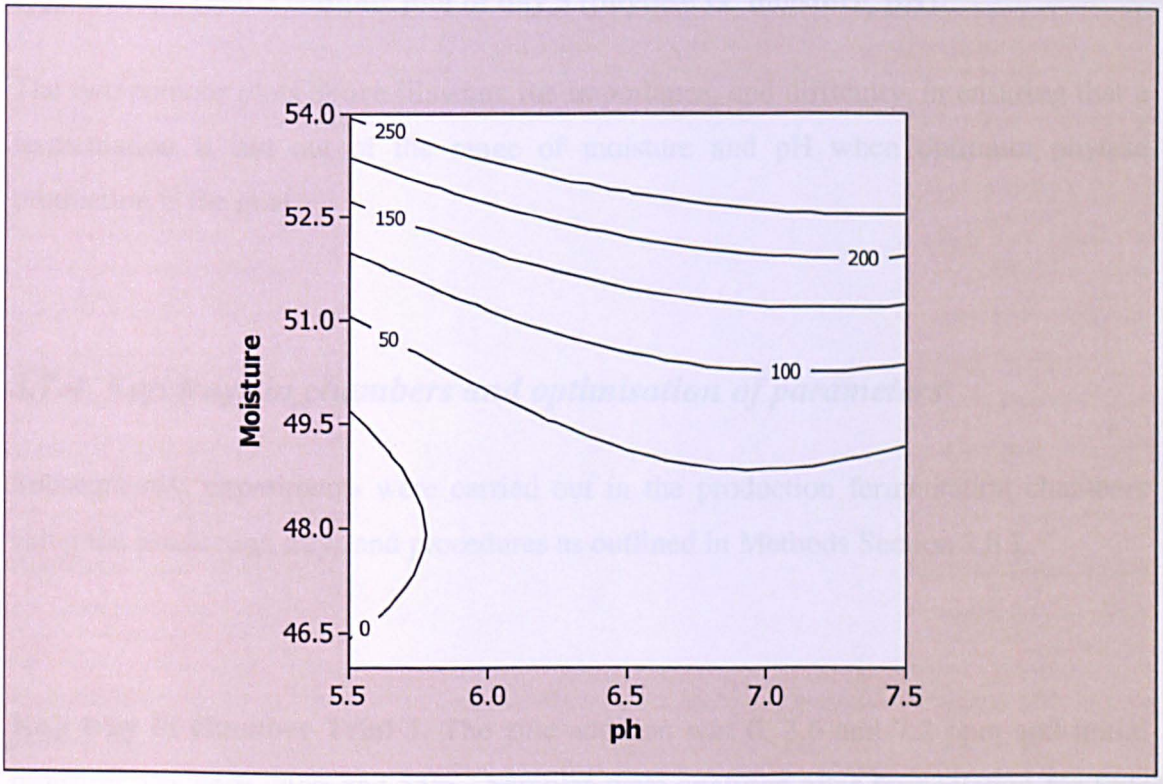


Figure 3.34 Trial 3 contour plot of day 4 (phytase versus moisture, pH).

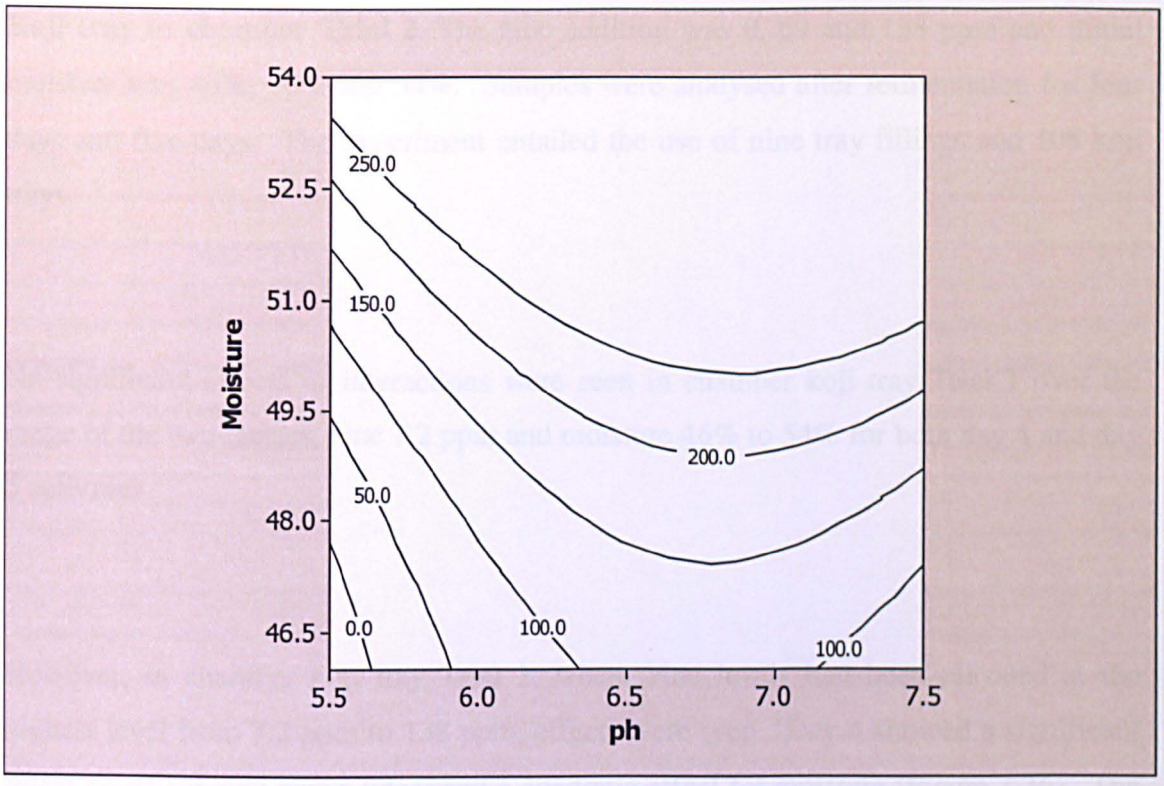


Figure 3.35 Trial 3 contour plot of day 5 (phytase vs. moisture, pH).

The two contour plots above illustrate the importance, and difficulty, in ensuring that a fermentation is not out of the range of moisture and pH when optimum phytase production is the goal.

3.7.4 Koji trays in chambers and optimisation of parameters

Subsequently, experiments were carried out in the production fermentation chambers using the production trays and procedures as outlined in Methods Section 2.8.2.

Koji tray in chamber Trial 1. The zinc addition was 0, 3.6 and 7.2 ppm and initial moisture was 46%, 50% and 54%. Samples were analysed after fermentation for four days and five days. The experiment entailed the use of nine tray fillings and 108 koji trays.

Koji tray in chamber Trial 2. The zinc addition was 0, 69 and 138 ppm and initial moisture was 46%, 50% and 54%. Samples were analysed after fermentation for four days and five days. The experiment entailed the use of nine tray fillings and 108 koji trays.

No significant effects or interactions were seen in chamber koji tray Trial 1 over the range of the two factors, zinc 7.2 ppm and moisture 46% to 54% for both day 4 and day 5 activities.

However, in chamber koji tray Trial 2, where zinc levels had been elevated at the highest level from 7.2 ppm to 138 ppm, effects were seen. Day 4 showed a significant linear effect for zinc plus a linear and a quadratic effect for moisture (Figure 3.36). The highest phytase results occurred at the lowest zinc level (0 addition) and at the lowest moisture (46%). On day 5, no significant effect of the two factors was seen. On day 5, moisture was at significance level of 0.104 suggesting that there was a 90% probability that there could be an effect, but the effect was negative. The effect was also negative in the day 4 results.

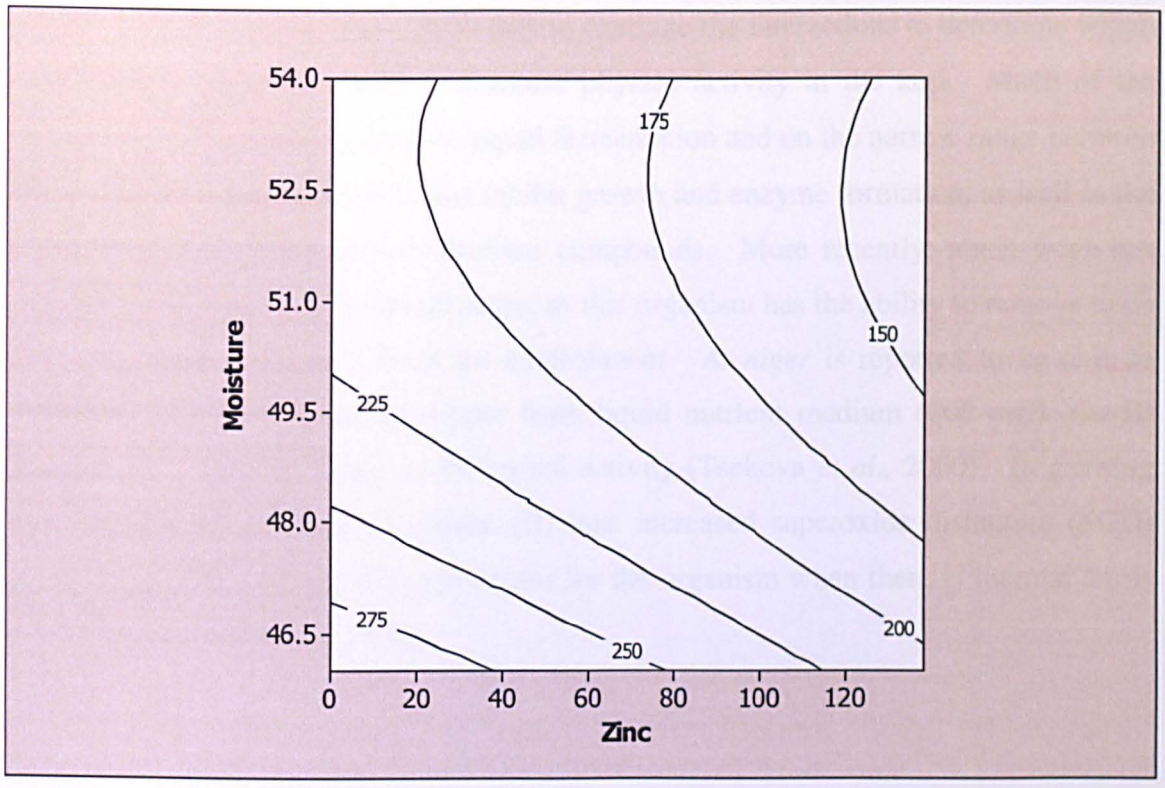


Figure 3.36 Chamber trial 2 contour plot of day 4. The % moisture versus ppm added zinc levels. Phytase activities (SPU/g) are illustrated on the lines.

3.7.5 Mineral supplementation (manganese, zinc and copper).

As early as 1931, McHargue and Calfee (1931) reported on the effects of manganese, copper and zinc on the growth and metabolism of Aspergillus and Rhizopus. At low levels they have been shown to exert a positive effect and at high levels they can exert toxic effects and the early reports already suggested that combinations of the optimum concentrations of manganese, copper, and zinc stimulated greater growth than did any one of these three metals alone. All three produced greater growth than did the combination of any two of these metals (McHargue and Calfee, 1931). In addition, these three metals also interact with the assimilation of other compounds in the growth medium (Bromberg *et al.*, 1997).

The use of multivariable experimentation for examining the effect of manganese, zinc,

copper and temperature was a good way to examine the interactions to determine which supplements might be useful to increase phytase activity in the koji. Much of the published research has focused on liquid fermentation and on the narrow range between levels that stimulate and levels that inhibit growth and enzyme formation, as well as the interactions with other growth medium compounds. More recently, much work has focused on *A. niger* and bioremediation, as this organism has the ability to remove toxic levels of copper and zinc from the environment. *A. niger* is reported to be able to accumulate large amounts of copper from liquid nutrient medium (200 mg/L Cu II) without the organism losing its biological activity (Tsekova *et al.*, 2000). In growing cell cultures the addition of copper (II) ions increased superoxide dismutase (SOD) activity and this compound is a protector for the organism when there is thermal stress (Tsekova and Todorova, 2002).

A series of preliminary experiments were conducted and Table 3.30 outlines the experimental design. The methodology was the standard method as described in Methods Section 2.4 – 2.8. The concentration units chosen were based on the nutrient database (Appendix D) that had been developed for the media ingredients. Since this was a preliminary experiment to explore the interactions, a low level was chosen and also an upper level 10 times higher, to force the culture to go in the direction of either good or poor phytase production. The temperatures of 30°C and 35°C were the ones routinely encountered during these fermentations.

Table 3.30 Koji flask trial 1 and 2. Additions of manganese, zinc, and copper to wheat bran.

Number	Mineral Additions			Temperature
	Mn	Zn	Cu	
Wheat bran Treatment 1	20 ppm	50 ppm	30 ppm	30°C
Wheat bran Treatment 2	200 ppm	50 ppm	30 ppm	35°C
Wheat bran Treatment 3	20 ppm	500 ppm	30 ppm	35°C
Wheat bran Treatment 4	200 ppm	500 ppm	30 ppm	30°C
Wheat bran Treatment 5	20 ppm	50 ppm	300 ppm	35°C
Wheat bran Treatment 6	200 ppm	50 ppm	300 ppm	30°C
Wheat bran Treatment 7	20 ppm	500 ppm	300 ppm	30°C
Wheat bran Treatment 8	200 ppm	500 ppm	300 ppm	35°C

These trials (Table 3.31 and Table 3.32) indicated that increasing the copper concentration in the medium from 30 ppm to 300 ppm had a positive effect on the phytase enzyme production (21 – 25 SPU/g). Manganese and zinc had a negative to poor effect. Temperature had a positive effect.

Table 3.31 Koji flask trial 1. Production of phytase activity under 8 treatment conditions.

	Trial 1 Phytase Activity (SPU/g)*			
Treatment**	Sample 1	Sample 2	Average	Std Dev
Treatment 1	428	357	393	50
Treatment 2	545	522	534	16
Treatment 3	514	607	561	66
Treatment 4	546	583	565	26
Treatment 5	727	639	683	62
Treatment 6	497	499	498	1
Treatment 7	425	487	456	44
Treatment 8	542	456	499	61

*Only two samples analysed from each trial since some flasks were contaminated and thus discarded and not analysed. ** Refer to Table 3.30 for treatment details.

Table 3.32 Koji flask trial 2. Production of phytase activity under 8 treatment conditions.

	Trial 2 Phytase Activity (SPU/g)*				
Treatment**	Sample 1	Sample 2	Sample 3	Average	Std Dev
Treatment 1	287	275	290	284	8
Treatment 2	323	388	276	329	56
Treatment 3	290	270	256	272	17
Treatment 4	288	326	221	278	53
Treatment 5	347	311	336	331	18
Treatment 6	261	274	222	252	27
Treatment 7	344	347	372	354	15
Treatment 8	339	360	312	337	24

Note: *Only two samples analysed from each trial since some flasks were contaminated and thus discarded and not analysed. ** Refer to Table 3.30 for treatment details.

Table 3.33 Multivariable trial 1. Mineral additions (factors).

Experiment	Results (SPU/g)	Experiment	Factors			
			A	B	C	D
Y1	393	1	-	-	-	-
Y2	533	2	+	-	-	+
Y3	561	3	-	+	-	+
Y4	564	4	+	+	-	-
Y5	683	5	-	-	+	+
Y6	498	6	+	-	+	-
Y7	456	7	-	+	+	-
Y8	499	8	+	+	+	+
Note: A= Manganese B= Zinc C = Copper D = Temperature						

Table 3.34 Multivariable trial 1. Mineral additions (effects).

	Phytase activity (SPU/g)				Effect
	Y1	Y2	Y3	Y4	
Effect A	140	3	-185	43	0.25
Effect B	168	31	-227	1	-6.75
Effect C	290	-35	-105	-65	21.25
Effect D	140	-3	185	43	91.25
Effect A-B	1063	1076	1017	1031	22.75
Effect A-C	997	954	1139	1097	-71.25
Effect A-D	1032	849	1244	1062	-106.25
Effect B-C	955	926	1181	1125	-106.25
Effect B-D	1060	891	1216	1020	-71.25
Effect C-D	1182	957	1094	954	22.75
Note: A= Manganese B= Zinc C = Copper D = Temperature					

Table 3.35 Multivariable trial 2. Mineral additions (factors).

Experiment	Results (SPU/g)	Experiment	Factors			
			A	B	C	D
Y1	284	1	-	-	-	-
Y2	329	2	+	-	-	+
Y3	272	3	-	+	-	+
Y4	278	4	+	+	-	-
Y5	332	5	-	-	+	+
Y6	252	6	+	-	+	-
Y7	354	7	-	+	+	-
Y8	337	8	+	+	+	+
Note: A= Manganese B= Zinc C = Copper D = Temperature						

Table 3.36 Multivariable trial 2. Mineral additions (effects).

	Phytase activity (SPU/g)				Effect
	Y1	Y2	Y3	Y4	
Effect A	45	6	-80	-17	-11.5
Effect B	-12	-51	22	85	11
Effect C	48	-77	82	59	28
Effect D	45	-6	80	-17	25.25
Effect A-B	615	616	626	581	6
Effect A-C	589	556	686	607	-37
Effect A-D	666	638	604	530	42.5
Effect B-C	691	613	584	550	42.5
Effect B-D	609	536	661	632	-37
Effect C-D	669	562	601	606	6
Note: A= Manganese B= Zinc C = Copper D = Temperature					

Although Table 3.33 to Table 3.36 did not demonstrate any statistically significant answers in terms of interactions between manganese, zinc, copper and temperature, in both sets of experiment, there were clearly copper and temperature effects. The data was further examined to look for interaction effects (Table 3.37).

Table 3.37 Analysis of data to determine interaction effects.

	-1	-1	-1	-1	393	284	338.5
	1	-1	-1	1	533	329	431.0
D =ABC	-1	1	-1	1	561	272	416.5
	1	1	-1	-1	564	278	421.0
Design	-1	-1	1	1	683	332	507.5
used	1	-1	1	-1	498	252	375.0
	-1	1	1	-1	456	354	405.0
	1	1	1	1	499	337	418.0

	-1	-1	-1	1
	1	-1	-1	-1
D =-ABC	-1	1	-1	-1
	1	1	-1	1
next	-1	-1	1	-1
suggested	1	-1	1	1

It should be noted that an interaction effect was used to account for the failure of the main effect to remain constant when the level of the second factor changes (for example when zinc is at -1, add 54.1 to the average of 58.4 and when zinc is at +1 subtract 54.1 from the average temperature effect).

Fractional experiments can be difficult to interpret when only half a fraction is run so the second fraction as outlined above (Table 3.37) should be run to complete the 16

trials.

Temperature and the zinc * temperature interactions were significant at the 0.01 level (one can be 90% sure that the interaction exists as there is less than a 10% probability that it is due to chance alone). When copper was omitted, the results became more significant as degrees of freedom for residual error increased from 1 to 4.

A plot of the data (Fig. 3.36) was carried out in factor space (a cube plot) using temperature, copper and zinc. The first two were used because they had the largest effects (i.e. changes going from a low level to a high level) and zinc because it appeared to interact with the first two factors.

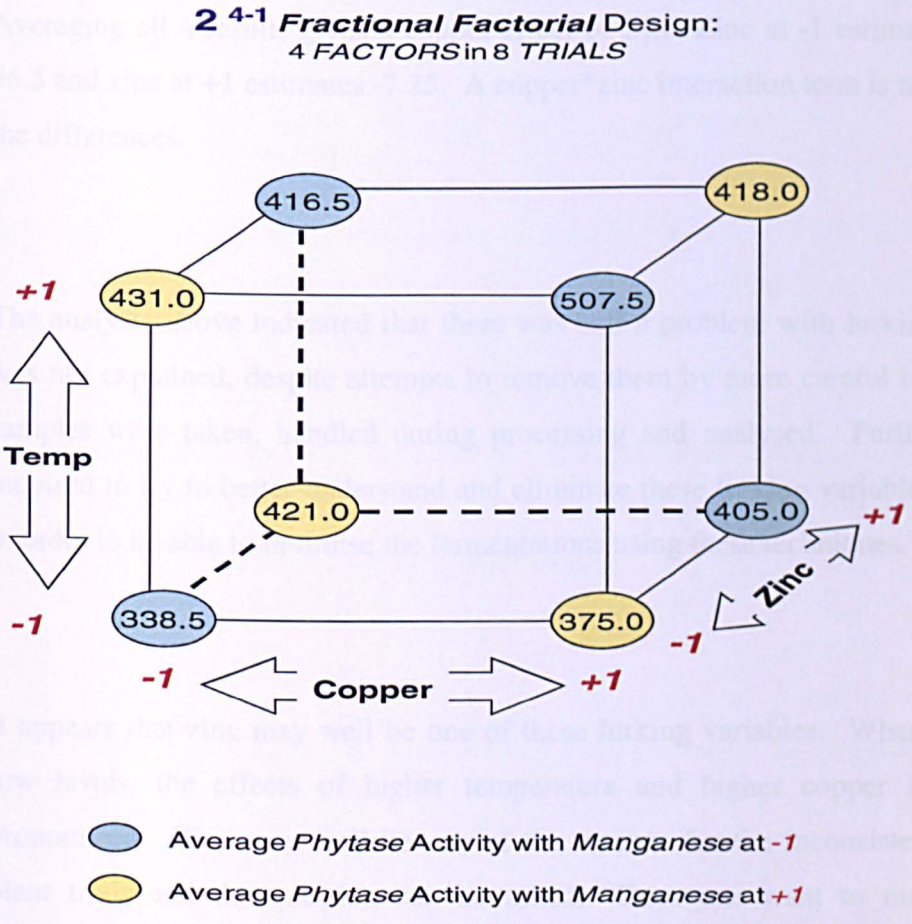


Figure 3.37 Factor space (cube plot) using temperature, copper and zinc.

Results at the far side of the cube represent the higher level of zinc (+1) and results at the front represent the lower level of zinc (-1). Four temperature effects were estimated by going from low (-1) to high (+1): 431.0 – 338.5 = 92.5 (zinc at -1) and 507.5 – 375.0 = 132.5 (zinc at -1); 416.5 – 421.0 = -4.5 (zinc at +1) and 418.0 – 405.0 = 13.0 (zinc at +1).

The averaging all 4 results gives a temperature effect of 58.4. Zinc at -1 estimates the effect as 112.5 and zinc at +1 estimates it at 4.25. A temperature * zinc interaction term is needed to explain the differences. Four copper effects were estimated by going from low (-1) to high (+1): 375.0 – 338.5 = 36.5 (zinc at -1) and 507.5 – 431.0 = 76.5 (zinc at -1);405.0 – 421.0 = -16.0 (zinc at +1) and 418.0 – 416.5 = 1.5 (zinc at +1).

Averaging all 4 results gives a copper effect of 24.6. Zinc at -1 estimates the effect as 56.5 and zinc at +1 estimates -7.25. A copper*zinc interaction term is needed to explain the differences.

The analysis above indicated that there was still a problem with lurking variables that was not explained, despite attempts to remove them by more careful treatment of how samples were taken, handled during processing and analysed. Further work is still required to try to better understand and eliminate these lurking variables in the process in order to be able to optimise the fermentations using these techniques.

It appears that zinc may well be one of these lurking variables. When it is present at low levels, the effects of higher temperature and higher copper levels are more pronounced. Zinc may well be one of the reasons for the inconsistent results in the plant trials and the question can be asked, "Does one want to maximise enzyme production but be willing to live with large fluctuations in production, or is the goal uniformity in terms of quality control for the customer?"

Often in complex systems with numerous variables, simpler experiments are relied upon in order to derive conclusions and modify processes. These results can sometimes lead to incorrect or incomplete conclusions that can mislead the experimenter. They can be very precise experiments without being accurate.

On the other hand, multivariable testing allows the experimenter to attribute the effects of the variables on the results. Multivariable testing can be a much more efficient means to gather information, albeit more complicated to design and execute. Multivariable experiments can run from a simple testing of three different variables with six possible combinations of variables, to more complex testing of hundreds of variables with billions of possible combinations. Additionally, multivariable testing requires much more complicated statistical models and the interpretation of the results can be complex in some situations.

Multivariable experimentation's main advantages focus on the speed and accuracy that can be achieved through their use. Many potential combinations of variables can be tested and a more holistic understanding of a system can be achieved. In addition, the time it takes to achieve results can be shortened. The negatives of this approach are mainly that it is more difficult to set up and complex to analyse. Nevertheless, if it is performed properly, improved results can be achieved with less experimentation, which can be costly, especially on an industrial scale.

The underlying rationale for these experiments lies in the theory that there exists an optimum at which the performance of the microorganism is at its peak for the desired project. Multiple optima may exist. In order to find the optima, all the levers in the system must be in operation and therefore multiple variables must be considered. Through complex statistical analyses, results can be obtained that indicate which direction to search, so that the distance to the optimum can be reduced. In biotechnological systems, this optimum can also change over time due to mutation,

especially with microorganisms that have undergone mutagenic treatment. Because of this potential, consistent monitoring of results, standard practices for strain maintenance, and ongoing research are critical for successful fermentation systems.

Chapter 4: Discussion

4.1 SSF Process

SSF technology, whilst being one of the oldest fermentation technologies has languished for a long time in the arena of being a niche or artisan type of production process and existed in the shadow of its big brother, submerged fermentation. SSF has not enjoyed the focus of some of the most talented technologists over the past century. Of late however, the interest level in this technology could not be much higher. Requests for articles from journals indicate interest in the subject and a solid-state review paper by Krishna (2005) was the most highly requested paper in 2006 from the journal Critical Reviews in Biotechnology (personal communication, G. G. Stewart, Editor, Critical Reviews in Biotechnology). This suggests that there is a surging interest in SSF systems and applications. As one reflects on SSF, and considers all the publications that come from all parts of the world and which cover every conceivable application, one is always struck with the question "Why has this technology not been vigorously embraced before?"

The answer to that question may lie in the distance between those conducting the research and those producing products using the technology. The research groups have not been successful in interesting commercial groups to make their ideas viable or they have not been able to start up commercial activities themselves. This lack of commercial success may have led to an impression that this technology is either outdated, or still not far enough developed for investors to show interest. Other technologies may be too entrenched and difficult to replace.

In this dissertation, an attempt has made to make the present system more consistent, effective and efficient. The studies in the literature have been primarily carried out using small laboratory scale units or pilot plants operated by research laboratories or institutes. While their findings are interesting, and often very useful in moving ideas for large-scale production forward, the distance between the laboratory bench and the production floor is much larger in SSF than in most fermentation technologies.

This is further compounded due to the minimal information that can be gleaned about production systems from private companies working in this area. It is a relatively secretive area and most companies tend to do all of their research in-house, investing significant amounts into this area and therefore jealously guarding their confidential data. For example, Amano Enzymes (Nagoya, Japan) have built a state of the art strain development research laboratory. The cost was over $100 million USD and their sales turnover the same year was $100 M USD (personal communication, Motoyuki Amano, Amano Enzymes). Shin Nihon Chemical Co. Ltd., (Aichi, Japan) built two bioreactors in the 1990's and these bioreactors cost more than their annual turnover. Many of the large companies are spending as much as 30% of their turnover on research and development efforts. Compared to other fermentation industries, such as brewing or the yeast production industry, difficulties are further enhanced because several different types of microorganisms are used on the different production systems with very different end products in mind. Very limited information can be gleaned from other processes even when openness exists, as there are so many parameters and conditions, which differ greatly. The high level of investment in this area further compounds the secretive nature of the business. These investments seem somewhat illogical when one thinks of the amount that could be saved by improved cooperation, but this further underlines the competitive nature of the business.

With this in mind, internal research must continue to be carried out and process improvement teams put in place. The SSF system discussed in this study has undergone considerable development based on the findings presented here. Cooling systems were improved, supplementation experiments were carried out and there was some success in improving fermentations, and lastly a new enzyme production unit was proposed, designed, and built.

Perhaps more significantly, the method for designing experiments and conducting them using multivariable methods will undoubtedly increase the rate of experimentation and therefore the speed at which results will be achieved and new findings can be

implemented.

Heat removal

The literature consistently states that heat removal is one of the most critical and most limiting aspects of SSF technology (Mitchell *et al.*, 2006). A better understanding of the mass and energy balance of the SSF system is presented in Section 3.1 and this review of the system allowed for a clearer idea of what some of the potential options could be for future systems. The system in use today relies upon water and air to remove the heat produced by the fermentation.

One concept to cool the fermentation chamber that has been proposed would be the use of evaporative cooling. This could be achieved by using dryer cool air, in conjunction with water cooling. The water evaporates on the trays and the koji is cooled in the trays in a more efficient manner. However, the product would experience some drying and this would require evaluation. This moisture could be returned by sprays or misting systems to the koji, but this added level of complexity may make this option unrealistic. With the importance that water activity plays in this fermentation, as discussed previously, Zweistra-Hoogschagen (2006) created a model for evaporative cooling and showed that for organisms that required a narrow temperature gradient, and had a high rate of heat production, evaporative cooling could be an effective way of controlling the fermentation. However, water activity had to be closely monitored, not only to keep removing heat, which Zweistra-Hoogschagen (2006) discusses, but also to keep the organism producing enzymes or any other desired product during the fermentation. Desiccation of fungal hyphae is one of the major challenges presented in the SSF system and Mitchell *et al.*, (2003) argue that it must be carefully controlled. There is a delicate cellular level balance, where there must be enough moisture to keep the hyphae moist, yet not too much, so that the aerial hyphae do not come in contact with the gas phase that is present throughout the empty spaces in the koji layer. If there is too much moisture present, this gas phase will not be present. In addition, higher levels of moisture may provoke increased levels of bacterial contamination. Evaporative cooling may not be the most preferential way to achieve the required cooling. This is not only

because of the drying issues, but also due to the difficulty in re-introduction of water once it has been removed. Re-introduction can give rise to the potential for pooling of condensed water on top of the koji mycelia mat, which can induce bacterial contamination, as bacteria will prefer the higher water activity in these areas.

The most practical solution, which is beneficial, efficient, and reliable, is to combine the rate of aeration and the moisture content of the substrate bed by passing humidified air through the system. This strategy, apart from controlling the temperature and limiting drying of the koji layer, maintains the moisture content of the substrate bed and also helps in superseding the oxygen demand of the fungus. There is some mention in the published literature and common knowledge within the industry that while the oxygen demand of a microorganism may be met, having more oxygen than required may alter some of the properties of the materials produced. It is thought that in this case side activities could vary if the oxygen level was higher than normal (personal communication, Frank Nalin, Lyven). Cooling the system with air would certainly also lower the requirements for water in the system, as well as the amount of energy that would be expended.

Process standardisation

One of the other more fundamental aspects of this study focused on consistency. A consistent process is critical to any process improvement or optimisation program. Past experiments had shown that false conclusions could be drawn due to complications in the fermentation system, or a lack of statistical modelling, or experimental design. Initially, eliminating inconsistencies in the system was important for future measurement reliability. By schematically studying the system and measuring the assays and measurements, it was possible to test the process and the measurements. Raw materials, particularly, the main raw material, wheat bran, were extensively reviewed. Wheat bran sampling proved that using the same supplier and monitoring their processes for consistency was important as variations between wheat bran sources

could significantly impact the final results of the fermentation. In addition, the consistency of the particle size, and the depth of the wheat bran, were both found to be important factors for successful fermentation. This appears logical as the gas transfer capabilities of the organism are an important factor for the growth of the fungal hyphae (Rahardjo *et al.*, 2002). Compaction of the bed, or heat build up within the koji layer, would considerably impact the fermentation.

Sampling techniques and assay reproducibility studies allowed a much higher level of confidence in the results. Standardising processes reduced the amount of variance between one batch and the next. Due to the nature of the koji material, mixing needs to be extremely standard and trials were conducted that employed coning and quartering.

One of the most difficult aspects of experimental work with solid-state media is the problems encountered in measuring biomass. Accurate biomass measurements would certainly help in the standardisation of many aspects of the process. In a liquid fermentation, separation between liquid and solid is easily accomplished, but the separation of solid from solid, especially when the mycelium of the organism has penetrated the medium, makes it extremely difficult to measure biomass (Mitchell *et al.*, 2006). As a consequence of the difficulty in separating the organism from the substrate, indirect growth measures are generally employed. Thus, it is necessary to either measure a biomass component that is not present in the uninoculated substrate (e.g. ergosterol or glucosamine) or to measure biomass activity (consumption of oxygen or production of carbon dioxide). Better techniques are needed to expand our understanding of the relationship between biomass production and enzyme production. New technologies continue to be examined in an attempt to devise a superior measuring system for the biomass. Digital processing is a technique that has recently been reported to give a good correlation, when used for indirect biomass estimation in a solid-state fermentation process, and it has the additional advantage of being a non-destructive technique (Couri *et al.*, 2006).

Understanding the level of variability in the system, allows for results that reliably show that effect, has been separated from noise present in the system. Significant levels of noise are present in the chamber system as shown in this study. It is believed that noise can be reduced even more in the future by strict control of process and sampling parameters. Substrate supplementation and advanced experimental design are tools that could help to find the key to more efficiency in the system and higher yields. Supplementation experiments that were carried out initially focused on nitrogen and carbon supplementation, followed by supplementation with single nutrients, followed by yeast extract and minerals, and finally the interaction between multiple variables was examined.

Side activities

One of the most interesting aspects of SSF is the high number and level of other enzymes valuable for animal feed nutrition that are present in the final dried koji product. In this study, we have referred to them as side activities, since they are not the main enzymes of interest. Animal trials have consistently shown a very significant benefit when the koji product was added to feed in terms of effect on the animal's health and nutrition (Ribeiro *et al.*, 2001; Wu *et al.*, 2003, Wu *et al.*, 2004a; Wu *et al.*, 2004b). It has been proposed that the side activities may hold the answer to the unique results of the products when they are applied to animal feed.

Four extracellular enzymes were routinely assayed, in addition to the two main enzymes of interest (i.e., protease and phytase). The data from 90 fermentations suggested that there was not a direct correlation between phytase production and these other enzymes. This was not an unexpected observation, as their functions in the organism are unique, although they are probably related in terms of the organism having to reach a certain growth stage before they can be secreted into the medium. It is well know that the organism produces a myriad of other enzymes that are beneficial, and are present in the final product, but at this time, what these enzymes are, and what specific effect and interactions they bring is an unexplored area.

Aspergilli contain a wide spectrum of enzymes for polysaccharide, protein, and lipid degradation. The two industrial Aspergilli, *A. niger* and *A. oryzae* contain the highest percentage of extracellular enzymes (Pel *et al.*, 2007). For example, a recent paper on *A. oryzae* has identified the plethora of enzymes that the organism produces (Kobayashi *et al.*, 2007).

A. oryzae compared to *Saccharomyces cerevisiae* or *Neurospora crassa,* is a species enriched with genes involved in biomass degradation and primary and secondary metabolism. The total number of *A. oryzae* genes that code for proteins longer than 100 amino acids is 12,074 (Kobayashi *et al.*, 2007). This species is used for saké fermentation due to the numerous amylolytic genes that it possesses, in addition to the numerous glucoamylase and α-glucosidase genes. Besides the amylolytic genes, it contains over 20 beta glucosidases and xylosidases and over 19 polygalacturonases. This species has a total of 69 genes for exopeptidase and 65 for endopeptidase.

There is a great opportunity for future research to determine which of the other enzymes secreted by the Aspergillus and Rhizopus strains possess this positive effect, when they act in synergy with the phytase and protease enzymes present in the feed additive product. Further investigation into why they may vary within a given fermentation is required. The individual activities were found to have a relationship between each other, but not a strictly linear one. A number of parameters will affect the microorganism's production of one enzyme over another. If this was better understood, different products could be tailored and tested for efficacy in their final applications.

The development of new strains of course offers one of the most significant opportunities for SSF technology. As seen in this study, extensive efforts were made to keep the system clean and to create optimum conditions for these sensitive organisms. If strains that are more robust could be identified, or strains that produced a higher yield of enzymes developed, the cost of the total process would probably be reduced

substantially.

In addition, the issue of genetic modification should be considered. If genetic modification is an option, the process could be shortened considerably. Difficulties associated with sporulation could be eliminated much more quickly than with UV mutagenesis (a very hit or miss strategy). Irrespective of the viewpoint taken on the opportunity for genetic modification, a major requirement is for faster techniques to screen potential candidate organisms. Today koji flask fermentations are carried out for strain examination and for treatment effects with those strains, such as different fermentation parameters or supplementation trials. These trials can be very laborious. Putting an experimental design in place for these trials, and continually searching for a more convenient technique for testing test SSF fermentations on a small scale, could speed up this process. One idea might be the use micro-fermentations, perhaps using gas permeable Petri dishes.

4.2 SSF System Comparisons

The conventional SSF system presented and used in this study certainly could be improved upon. There are many different options for this development, from incremental improvement to complete conversion to a new system. The challenge today for SSF revolves primarily around the issues just discussed and the high level of labour utilised in the plant. For a plant that produces 100 tons of product, roughly 100 people are required. This is far more than would be necessary for a submerged fermentation system with a similar output. Most of the tasks carried out are quite basic and include material handling, movement of trays and trolleys, and washing of materials and fermentation chambers. Automation of the process would have the benefit of limiting human contact with the product and therefore reducing the potential for contamination.

If the trays and trolleys could be semi-automated, a significant portion of the labour would be removed. Tray handling would involve systems for washing, stacking and de-stacking, filling, loading onto trolleys or moving into the fermentation space, removal of

product, and empty tray return to washing. A system of conveyors could be devised. However, trays are expensive especially when such large quantities are required. In Serdán there are currently over 30,000 trays. The space required for housing the fermentation is also very significant. This is partially explained by the height that can be used for the fermentation. It is limited by the height a human can reach to fill a trolley. Automation of the trolley filling process would remove this limitation.

If looked at in a broad sense, there is no reason why SSF should not be as competitive as submerged fermentation on a cost per unit basis. Today SSF's advantage is largely based on the use of alternative raw materials and the production of superior and unique final products. Systems exist that superficially would have a lot in common with SSF. One of these is the malting process used by the brewing and distilling industries. Mimicking a process such as malting would allow for better automation and handling of the fermentation material. The malting process deals with a lot of the same issues as the SSF process. Heat development is an issue, as well as air and moisture levels and material handling. However, malting has been developed into a far less laborious process than it was just a few decades ago. A modern malting plant requires very little labour and can handle thousands of tons of product through the highly controlled process. The cost of malt is around $250 USD/ton. This approximate cost would be extremely competitive in the enzyme market, even if there were many other costs that would be taken on by SSF production over malt production. Taking the SSF process and adapting it would present some challenges. The agitation required in turning the malt bed would not be needed or even desired for SSF, as it would disturb the organism and break mycelia and thus hamper growth. However, the high moisture aeration systems that cool the barley during malting would certainly be beneficial to SSF. The main difference with SSF is the sensitivity to contamination. Malt also can have this problem, but not to the same degree. Also, even with fungi that grow very vigorously such as *R. oligosporus,* that can create almost a mat of material, the initial material in the fermentation usually is very fine in texture, and this would be more difficult to move than barley.

Other options of course include bioreactors. Compared to tray systems, they still

require a lot of capital investment and thus far do not appear to give the return on investment. The metabolite produced from the process would have to be a high value product for the bioreactors to be cost effective. The need for special handling would give bioreactors an economic advantage over tray systems. The cost of a one ton bioreactor is estimated to be between $250,000 and $1,000,000 USD. If it costs $400,000 USD and it produces one ton every three to four days for a year, that would be roughly 100 tons per year. That would require $400 USD /ton in capital cost if it had a 10 year lifespan. Depending on the industry, that could represent a prohibitive cost to put onto a product. Another aspect would be the number of units that would be required. The volumes in most bioreactors would be less than one ton at a time and with a few exceptions, it might be as high as five tons per batch. For the cost of the system, tray plants could be built that have more flexibility, especially if they are in low labour cost countries.

One way around this issue could be to increase the volumes that can be handled in these systems. Using microorganisms that are do not require such a long fermentation might be one way to increase the throughput in a reactor. If the bed depth in a bioreactor could be increased, this would be another option.

The SSF process produces products with a unique enzyme range and the products are substantially different from what is produced by submerged fermentation. A great many opportunities and challenges lie ahead in this area, and it will be one of increased research activity in the near future.

4.3 Opportunities for Applications of SSF

SSF systems have tremendous potential for the future. The technology is only now being moved into areas where it can flourish outside of food production and niche enzyme applications. With so many new issues arising in the areas of bioenergy production, food production, and the environment, this technology provides potential solutions that may placate varying concerns from very disparate industries. The energy,

feed, and food industries do not have an existing relationship or dialogue and predominately each would see environmental issues as an area that would pose a threat to their businesses, not an area where business or technology opportunities might lie. The present situation may hold promise for more alignment between these differing groups and their agendas. Often when vastly different groups have the chance to work together, the greatest strides are made. It may be that the pressure on the system today, will create more potential for cooperation and that technology may hold the key to this opportunity.

4.3.1 SSF's unique solution for animal feed

In terms of the animal feed industry, the clear benefit of SSF can be seen in the fermentation of materials that today have little or no value. Many by-products and waste materials could be improved in terms of their nutritional value through an SSF process.

With the increase in the production of ethanol, such waste products are distiller's dried grains with solubles (DDGS) coming from the distilling industry and spent grains from brewing. The volumes of DDGS in the market are steadily increasing. DDGS when it leaves the distillery makes up the same volume as one third of the total corn going into the distillery. The other two thirds are converted to ethanol and carbon dioxide and fibrous material remains. It varies significantly in terms of its nutrient levels depending on the fermentation and distilling process and often contains high levels of mycotoxins due to improper storage and handling.

Using a SSF enzyme in a diet that contains high levels of DDGS can mitigate these negative properties of the material and release more nutrients. DDGS is a less expensive material and therefore there is currently great interest in using it. Although previously just used for ruminant animals, DDGS is now utilised by mono-gastric animals at relatively high levels, as much as 26% of the diet with an SSF enzyme (Pierce et al., 2007). Animal nutritionists are finding not only similar results to standard

diets, but improvements in overall growth and growth efficiency.

Fermenting DDGS and other similar materials in a SSF system could further add value to the materials and improve their use in agricultural systems. With the use of specialised strains and modified processes, these materials can be improved simply by increasing the protein level, or to a more advanced level by producing metabolites and enzymes. There is currently great interest in achieving the highest level of integration possible. Some distilleries wish to integrate locally and work with, or become, cattle producers. They would utilise the locally grown corn and feed the DDGS to cattle. In turn, the manure will be burned in order to create energy which will power the plant. If SSF technology was used, the grains would be much more suitable for the animals and they would not be eating a waste stream, but rather an added value product, which could help to improve the quality of the meat, shorten time to slaughter, and improve their immune status.

With the backdrop of food safety, many of these feeding issues can be solved through greater traceability and cooperation. The pet food scare brought food safety into greater focus than possibly any other food or feed scare in the recent past (The Economist, 2007b). It also brought the reputation of one country (China) into such question, that the head of their FDA was put to death for accepting bribes that may have led to filler material being used which contained toxins (Kahn, 2007). So important is food in this globalised and shrinking world that these situations can occur so rapidly. The consequences for unlawful activity are severe. The animal feed industry and its suppliers are under tighter scrutiny and more pressure than ever before and the best way forward may involve more localised production and completely traceable materials. SSF's ability to upgrade materials, means that localised production and utilisation may be as competitive as global sourcing.

Taking this idea further, SSF can play an even more vital role in the biorefinery concept, which tailors the waste stream for downstream processing by SSF and can

either feed that material back into the alcohol fermenter, as an enzyme for further alcohol production, or sell it as an enzyme or animal feed additive, depending on the organism used and the enzymes produced in the fermentation. The biorefinery concept is an area of potential development. Through the use of SSF, cellulose can be enzymically hydrolysed and therefore help with the conversion of cellulose into energy. In a recent National Geographic publication (Bourne, 2007), ethanol from corn was compared to ethanol from sugar cane. According to the study, in order to make ethanol from corn, one unit of energy is needed for every 1.3 units ethanol produced. For ethanol made from sugar cane, one unit of input yields eight units of energy. Sugar cane ethanol also produces 56% less greenhouse gases than gasoline, whilst corn based ethanol produces 22% less than gasoline. Because of this, corn based ethanol producers must develop novel ways to find efficiencies and SSF may be a logical way to accomplish this to produce value added products. Using raw materials such as corn stover or corn cobs could be a way of utilising everything that comes from the field and SSF may hold even great opportunities in the area of energy production. As energy prices increased in the summer of 2006, many thought that the time for alternatives fuels had finally arrived. However, prices retreated slightly and at the same time, so did many of the subsidies that supported the ethanol producer. Recently however, the world price of crude oil has increased again to record levels, and the time may be opportune for alternative biofuels that employ SSF during their production.

Appendix A

Chapter 5: Appendices

Appendix A Strain identification of contaminating microorganisms (referenced in Section 2.7.7)

External Lab ID	*Assay*	*Method used*	*Result*	*ID Probability*
251-07	*Bacterial Identification*	*Biolog, automated system Microplate GP2*	*Paenibacillus polymyxa*	*99%*
252-07	*Bacterial Identification*	*Biolog, automated system Microplate GP2*	*Bacillus megaterium A*	*19%*
253-07	*Bacterial Identification*	*Biolog, automated system Microplate GP2*	*Micrococcus luteus (ATCC 9341)*	*99%*
254-07	*Bacterial Identification*	*Biolog, automated system Microplate GP2*	*Cellulomonas hominis (CDC-3)*	*99%*
256-07	*Bacterial Identification*	*Biolog, automated system Microplate GP2*	*Bacillus subtilis C*	*97%*
257-07	*Bacterial Identification*	*Biolog, automated system Microplate GP2*	*Cellulosimicrobium*	*95%*
259-07	*Bacterial Identification*	*Biolog, automated system Microplate GP2*	*Micrococcus luteus (ATCC 9341)*	*99%*
260-07	*Bacterial Identification*	*Biolog, automated system Microplate GP2*	*Alloiococcus otitis*	*99%*

Appendix A Strain identification of contaminating microorganisms (referenced in Section 2.7.7)

Strain Identification of isolated microorganisms in process line

Tube No.	External Lab ID	Assay	Method used	Result	ID probability	Observations from Alltech Serdán Lab
1	251-07	Bacterial Identification	Biolog, automated system Microplate GP2	Paenibacillus polymyxa	99%	Isolated from contamination
2	252-07	Bacterial Identification	Biolog, automated system Microplate GP2	Bacillus megaterium A	19%	Isolated from contamination
3	253-07	Bacterial Identification	Biolog, automated system Microplate GP2	Micrococcus luteus (ATCC 9341)	99%	Isolated from contamination
4	254-07	Bacterial Identification	Biolog, automated system Microplate GP2	Cellulomonas hominis (CDC-3)	99%	Isolated from contamination
5	255-07	Bacterial Identification	Biolog, automated system Microplate GP2	Not identified under 1500 species	-	Isolated from contamination
6	256-07	Bacterial Identification	Biolog, automated system Microplate GP2	Bacillus subtilis C	97%	Isolated from contamination

Appendix A Strain identification of contaminating microorganisms (referenced in Section 2.7.7.)

Tube No.	External Lab ID	Assay	Method used	Result	ID probability	Observations from Alltech Serdán Lab
7	257-07	Bacterial Identification	Biolog, automated system Microplate GP2	Cellulosimicrobium	95%	Isolated from contamination
8	258-07	Bacterial Identification	Biolog, automated system Microplate GP2	Not identified under 1500 species	-	Isolated from contamination
9	259-07	Bacterial Identification	Biolog, automated system Microplate GP2	Micrococcus luteus (ATCC 9341)	99%	Isolated from contamination
10	260-07	Bacterial Identification	Biolog, automated system Microplate GP2	Alloiococcus otitis	99%	Isolated from contamination
11	261-07	Fungal Identification	Biolog, automated system Microplate FF	Rhizopus spp.	No reported	Protease strain used Production
12	262-07	Fungal Identification	Biolog, automated system Microplate FF	Aspergillus spp.	No reported	Phytase strain used in production
13	263-07	Fungal Identification	Biolog, automated system Microplate FF	Not identified in database	-	Orange Fungus contamination

Appendix B

Appendix B Mass balance data and equations (referenced in Section 3.1)

Air flows, humidity, and temperatures and water flows and temperatures for section 3.1. Fermentation time: 4 days

		Air inlet			Air outlet		Water flow		
Day	Time (h)	Velocity (m/s)	Humidity (%)	Temperature (°C)	Humidity (%)	Temperature (°C)	Flow (l/s)	Temperature (°C) $_{inlet}$	Temperature (°C) $_{outlet}$
1	0	4.0	60.1	20.0	89	22.3	0.00	20	29.2
1	2	4.6	68.1	16.2	89	26.1	0.25	43	29.3
1	4	2.3	69.3	19.4	100	30.7	0.22	33	29.3
1	6	2.2	68.6	18.2	100	28.9	0.00	23	32.5
1	8	2.0	67.1	17.9	100	27.8	0.10	27	30.0
1	10	2.2	65.4	17.4	100	28.2	0.14	24	29.0
1	12	2.4	62.3	16.5	100	27.5	0.03	50	29.0
1	14	2.6	57.5	15.8	100	28.3	0.00	25	29.1
1	16	3.6	55.8	16.7	100	29.8	0.00	25	28.6
1	18	3.1	58.1	19.9	100	29.0	0.06	50	29.6
1	20	4.3	56.4	20.1	100	28.9	0.00	25	29.1
1	22	2.4	56.8	19.9	100	29.0	0.00	27	29.1
1	24	3.6	59.1	18.2	100	29.1	0.01	25	29.1
2	26	3.1	64.9	16.5	100	28.9	0.12	28	29.1
2	28	4.0	61.4	16.2	100	28.0	0.00	30	29.1
2	30	3.5	62.5	16.4	100	28.4	0.00	25	32.1
2	32	3.8	60.1	16.0	100	27.8	0.00	45	30.1
2	34	3.5	58.3	15.8	100	29.3	0.07	31	33.3
2	36	3.6	55.6	16.4	100	28.1	0.00	25	31.3
2	38	2.8	50.3	17.1	100	29.0	0.05	27	32.3
2	40	3.0	48.9	18.9	100	29.2	0.31	31	33.8
2	42	2.9	45.0	21.3	100	29.3	0.00	20	32.3
2	44	3.1	48.9	20.5	100	30.1	0.00	25	35.8
2	46	3.2	53.6	19.9	100	29.4	0.00	25	38.9
2	48	3.4	65.8	18.3	100	28.2	0.28	28	28.3
3	50	4.3	65.1	16.8	100	28.9	0.22	29	31.4
3	52	2.8	57.5	16.9	100	28.3	0.21	27	32.3
3	54	2.3	64.1	16.5	100	29.1	0.06	25	35.4
3	56	2.1	67.1	17.9	100	28.2	0.15	23	33.9
3	58	2.5	59.9	18.2	100	26.2	0.11	19	41.1
3	60	2.3	58.2	18.4	100	23.4	0.25	23	32.2
3	62	1.5	56.1	21.5	100	24.4	0.25	21	32.1
3	64	2.3	45.9	23.5	100	28.5	0.11	17	36.4
3	66	3.2	44.7	23.7	100	29.0	0.12	25	39.0
3	68	3.7	67.1	18.9	100	27.7	0.16	24	39.5
3	70	3.5	68.3	17.1	100	25.3	0.08	18	43.5
3	72	3.6	65.8	18.3	100	29.1	0.35	21	26.3
4	74	3.1	65.1	16.8	100	28.2	0.11	18	35.0
4	76	3.5	55.2	16.9	100	26.3	0.08	22	31.5
4	78	3.2	65.2	16.5	100	25.2	0.00	18	37.9
4	80	3.5	66.2	17.9	100	24.4	0.00	18	40.8
4	82	3.7	65.3	18.5	100	28.5	0.00	18	42.3
4	84	3.5	66.7	20.3	100	29.0	0.00	18	36.9
4	86	2.9	55.3	21.5	100	27.7	0.00	18	37.3
4	88	3.1	45.9	23.5	100	25.3	0.00	21	38.2
4	90	3.2	44.7	23.7	100	26.2	0.00	21	37.6
4	92	3.4	67.1	18.9	100	24.3	0.00	21	42.3
4	94	3.5	68.3	17.1	100	21.3	0.00	21	43.9
4	96	3.7	69.3	18.3	100	22.1	0.00	21	38.1

1 In order to calculate the heat flow in the fermentation chamber released by the substrate during fermentation, the following variables need to be measured.

$$\bar{v}_{L1}\, T_{L1}, T_{L2}, T_{w3}, T_{w4}, \dot{m}_{w3}, \varphi_1, \varphi_2$$

2 Determination of the specific humidity of air by measuring the moisture content

If the relative humidity is known, the specific humidity of air can be calculated as follows:

$$x = x'(T_L) \cdot \varphi \cdot \frac{p - p'(T_L)}{p - \varphi \cdot p'(T_L)}$$

3 Determination of $\dot{m}_L$ by measuring the velocity of air $\bar{v}_L$ entering the chambers

$\dot{V}_L$ can be calculated from $\bar{v}_L$ as follows:

$$\dot{V}_L = \bar{v}_L \cdot \frac{d^2}{4} \cdot \pi$$

The density as a function of temperature and relative humidity of the air can be calculated as follows:

$$\rho_L(T_L, \varphi) = \frac{p_0}{R_L \cdot T_L} \cdot \left(1 - 0.378 \cdot \frac{p'(T_L) \cdot \varphi}{p_0}\right)$$

Mass flow of air is a function of ρ_L and $\dot{V}_L$

$$\dot{m}_L = \rho(T_L, \varphi) \cdot \dot{V}_L$$

4 Determination of $\dot{m}_{w3}$ by measuring the volume flow $\dot{V}w_3$

The mass flow of water, as a function of its specific volume $v_w^{'}$ and volume flow rate $\dot{V}_{w3}$, can be calculated as follows:

$$\dot{m}_{w3} = \frac{\dot{V}_{w3}}{v_w^{'}(T_{w3})}$$

5 Calculation of $\dot{m}_{w4}$ from water balance

$$\dot{m}_{w4} = \dot{m}_{w3} - \dot{m}_{L1}(x_2 - x_1)$$

6 Calculation of specific enthalpies

Specific enthalpy: $\left(h_{1+x}\right)_i = c_{pL} \cdot T_{L,i} + x_{L,i}\left(r_v + c_{pd} \cdot T_{L,i}\right);$

$$h_{w,i} = c_{pw} \cdot T_{w,i}$$

Abbreviations

The values of p'(T), and x'(T) can be obtained from Tables 1 and v' from Table 2.

The values of p'(T), and x'(T)

Symbol	Description	Unit
x	Specific humidity	$\frac{g_{H20}}{kg_{d.air}}$
x'	Specific humidity at saturation	$\frac{g_{H20}}{kg_{d.air}}$
φ	Relative humidity	%
$p'(T_L)$	Saturation pressure of water vapor	Pa
c_{pL}	Specific heat capacity of dry air	$\frac{kJ}{kg \cdot K}$
c_w	Specific heat capacity of water	$\frac{kJ}{kg \cdot K}$
c_{pd}	Specific heat capacity of water steam	$\frac{kJ}{kg \cdot K}$
r_v	Enthalpy of steam	$\frac{kJ}{kg}$
R_L	Gas constant of dry air	$\frac{J}{kg \cdot K}$
ρ	Density	$\frac{kg}{m^3}$
$\bar{v}_L$	Velocity of air which enters the chambers	$\frac{m}{s}$

8 Constants used for calculations

$$c_{pL} = 1.006 \frac{kJ}{kg \cdot K}; r_v = 2502 \frac{kJ}{kg}; c_{pd} = 1.86 \frac{kJ}{kg \cdot K}; c_w = 4.19 \frac{kJ}{kg \cdot K}; R_L = 287 \frac{J}{kg \cdot K}$$

$$p_0 = 78500 Pa$$

Appendix C

Appendix C Wheat Bran Analysis (referenced in Section 3.5.1)

Wheat Bran Analysis

Source	Calcium (%)	Copper (ppm)	Iron (ppm)	Magnesium (%)	Manganese (ppm)	Phosphorous (%)	Potassium (%)
Production Wheat Bran	0.11	13.00	161.00	0.45	163.00	1.11	1.34
El Paraiso	01-01						
La Espiga	0.18	14.00	213.00	0.46	161.00	1.13	1.31
San Blas	0.16	13.00	123.00	0.40	145.00	1.01	1.23
La Asuncion	0.85	18.00	156.00	0.55	159.00	1.28	1.36

Source	Sodium (%)	Sulfur (%)	Zinc (ppm)	Detergent Fibre (%)	Ash (%)	Crude Fat (%)	Energy (Mcal/lb)
Production Wheat Bran	0.02	0.19	98.00	13.00	5.43	3.15	1.63
El Paraiso							
La Espiga	0.02	0.20	103.00	12.10	5.07	4.39	1.67
San Blas	<0.01	0.19	85.00	14.10	4.64	3.54	1.65
La Asuncion	<0.01	0.18	90.00	14.60	6.61	3.62	1.61

Source	Dry Matter (%)	Energy (Mcal/lb)	Moisture (%)	Gain (Mcal/b)	Lactation (</cal/lb)	Maint (Mcal/lb)	Protein (%)	Digestibl e nutrient
Production Wheat Bran	88.87	1.50	11.13	0.59	0.85	0.88	18.40	81.30
El Paraiso								
La Espiga	88.77	1.55	11.23	0.61	0.87	0.91	16.90	83.50
San Blas	87.96	1.53	12.04	0.60	0.86	0.89	16.70	82.40
La Asuncion	89.20	1.49	10.80	0.58	0.84	0.87	16.20	80.60

Appendix D

Appendix D Nutrient database (referenced in Section 3.7.5)

Source	Calcium (%)	Copper (ppm)	Iron (ppm)	Magnesium (%)	Manganese (ppm)	Phosphorous (%)	Potassium (%)	Sodium (%)
Wheat Bran	0.11	13.00	161.00	0.45	163.00	1.11	1.34	0.02
CaCl2. H2O	34.50	0.00	0.00	0.03	0.00	0.00	0.84	0.84
MgSO4.7H2O	0.14	0.00	966.00	9.64	92.00	0.00	0.11	0.09
KH2PO4	0.00	0.00	0.00	0.00	0.00	22.28	28.00	0.05
KCl	0.00	0.00	0.00	0.04	0.00	0.00	51.60	0.48
Glucose	<0.01	<20	<50	<0.01	<20	<0.05	<0.05	<0.01
Glucose								
Yeast Extract	0.08	<1	24.00	0.17	1.00	1.83	3.36	2.61
Starch	<0.01	<20	<50	<0.01	<20	<0.05	<0.05	<0.01
Starch								
Wheat Bran - El Paraiso								
Wheat Bran - La Espiga	0.18	14.00	213.00	0.46	161.00	1.13	1.31	0.02
Wheat Bran - San Blas	0.16	13.00	123.00	0.40	145.00	1.01	1.23	< 0.01
Wheat Bran - La Asuncion	0.85	18.00	156.00	0.55	159.00	1.28	1.36	< 0.01
Phytase 3A1	1.43	< 1	< 5	0.55	67.00	1.57	3.04	1.09
Phytase 3A2	1.48	< 1	< 5	0.54	63.00	1.58	3.00	1.11
Phytase 3B	0.92	< 1	< 5	0.42	41.00	1.11	2.57	0.92
Phytase 4D1	0.69	< 1	< 5	0.34	64.00	0.95	1.74	0.47
Phytase 4D2	0.77	< 1	118.00	0.39	74.00	1.00	1.79	0.56
Phytase TD1	1.13	62.00	< 5	< 0.01	124.00	1.10	1.84	0.58
Phytase TD2	0.71	< 1	< 5	< 0.01	72.00	0.83	1.28	0.46
3SC-22	1.14	< 1	101	0.36	50	1.14	1.9	0.66
4SD-22	0.68	< 1	98	0.34	60	0.9	1.51	0.46
TD1-22	**1.53**	**142**	**< 5**	**< 0.01**	**190**	**1.26**	**2.42**	**< 0.01**
3SC-23	0.74	< 1	< 5	0.39	34	1.08	2.5	0.88
3SF-23	0.91	< 1	< 5	0.37	38	0.99	2.04	0.76
4SB-23	0.61	< 1	99	0.35	57	0.92	1.61	0.5
4SA-23	0.58	< 1	< 5	0.35	54	0.9	1.7	0.51
TD3-23	0.71	< 1	< 5	< 0.01	55	0.84	1.48	0.47
TD2-23	0.92	< 1	< 5	< 0.01	73	0.98	1.84	0.55
3SB-24	1.18	< 1	< 5	0.4	33	1.12	2.3	0.91
3SA-24	1.01	< 1	< 5	0.42	42	1.1	2.32	0.87
4SD-24	0.71	< 1	109	0.35	63	0.93	1.59	0.46
4SA-24	0.64	< 1	92	0.32	54	0.85	1.45	0.45
TD2-24	0.76	< 1	< 5	< 0.01	56	0.93	1.65	0.53
TD1-24	0.81	< 1	< 5	< 0.01	63	0.87	1.86	< 0.01
3SF-25	1.09	< 1	< 5	0.4	43	1.13	2.16	0.81
4SD-25	0.70	< 1	115.00	0.38	65.00	0.98	1.69	0.51
TD-25	0.90	< 1	< 5	< 0.01	73.00	1.08	2.06	< 0.01

Source	Sulfur (%)	Zinc (ppm)	Acid detergent fiber (%)	Ash (%)	Crude fat (%)	Digestable energy (Mcal/lb)	Dry matter (%)	Metabolized energy (Mcal/lb)
Wheat Bran	0.19	98.00	13.00	5.43	3.15	1.63	88.87	1.50
CaCl2. H2O	0.00	0.00	0.00	94.20	0.55		100.00	
MgSO4.7H2O	12.70	52.00	0.90	46.50	0.28		59.30	
KH2PO4	0.00	0.00	0.00	86.40	0.00		99.64	
KCl	0.05	23.00	0.00	99.50	0.00		99.71	
Glucose	<0.05	<20	<0.5	1.48	<0.1	1.77	90.66	1.70
Glucose								
Yeast Extract	1.25	22.00	0.68	15.50	0.27	1.30	93.85	1.07
Starch	<0.05	<20	<50	<0.1	0.73	1.81	87.45	1.74
Starch								
Wheat Bran - El Paraiso								
Wheat Bran - La Espiga	0.20	103.00	12.10	5.07	4.39	1.67	88.77	1.55
Wheat Bran - San Blas	0.19	85.00	14.10	4.64	3.54	1.65	87.96	1.53
Wheat Bran - La Asuncion	0.18	90.00	14.60	6.61	3.62	1.61	89.20	1.49
Phytase 3A1	0.92	50.00	< 0.50	15.60		1.45	3.94	1.30
Phytase 3A2	0.92	47.00	< 0.50	12.00		1.43	3.98	1.28
Phytase 3B	0.75	35.00	< 0.50	13.10		1.53	5.21	1.39
Phytase 4D1	0.44	46.00	< 0.50	6.85		1.70	5.74	1.57
Phytase 4D2	0.50	66.00	< 0.50	10.20		1.58	5.33	1.45
Phytase TD1	<0.01	93.00	< 0.50	13.30		2.37	1.93	2.19
Phytase TD2	<0.01	58.00	< 0.50	19.80		1.77	2.38	1.64
3SC-22	0.65	38	< 0.50	12		1.48	5.7	1.35
4SD-22	0.44	44	< 0.50	10.4		1.69	5.8	1.56
TD1-22	**<0.01**	**190**	**< 0.50**	**31.8**		**1.46**	**1.31**	**1.35**
3SC-23	0.76	34	< 0.50	17		1.5	5.41	1.37
3SF-23	0.67	38	< 0.50	11.7		1.58	6.1	1.45
4SB-23	0.47	47	< 0.50	8.22		1.62	6.51	1.5
4SA-23	0.46	42	< 0.50	5.38		1.69	5.65	1.56
TD3-23	<0.01	55	< 0.50	< .1		1.82	2.33	1.69
TD2-23	<0.01	59	< 0.50	< .1		1.79	2.07	1.67
3SB-24	0.78	33	< 0.50	11.5		1.55	3.94	1.41
3SA-24	0.75	42	< 0.50	13.3		1.41	4.46	1.29
4SD-24	0.44	51	12	8.5		1.53	5.73	1.42
4SA-24	0.41	38	< 0.50	8.94		1.61	5.55	1.49
TD2-24	0.44	56	23.3	14		1.34	2.28	1.24
TD1-24	<0.01	63	< 0.50	7.95		1.45	2	1.34
3SF-25	0.7	37	< 0.50	12.7		1.53	5.29	1.4
4SD-25	0.47	60.00	< 0.50	11.40		1.50	5.59	1.38
TD-25	<0.01	73.00	38.20	9.57		1.32	1.41	1.22

Source	Moisture (%)	Net energy gain (Mcal/lb)	Net-energy-lactation (Mcal/lb)	Net energy-maint(Mcal/lb)2	Crude protein (%)	Total digestible nutrient (Mcal/lb)	Aflatoxin B1 (limit 5 ppb)
Wheat Bran	11.13	0.59	0.85	0.88	18.40	81.30	0.00
CaCl2. H2O	0.00				2.24		
MgSO4.7H2O	40.70				0.00		
KH2PO4	0.36				0.00		
KCl	0.29				0.00		
Glucose	9.34	0.65	0.93	0.98	<0.2	88.70	
Glucose							
Yeast Extract	6.15	0.39	0.67	0.66	66.30	65.90	
Starch	12.55	0.66	0.95	1.00	0.52	90.60	
Starch							
Wheat Bran - El Paraiso							0.00
Wheat Bran - La Espiga	11.23	0.61	0.87	0.91	16.90	83.50	0.00
Wheat Bran - San Blas	12.04	0.60	0.86	0.89	16.70	82.40	0.00
Wheat Bran - La Asuncion	10.80	0.58	0.84	0.87	16.20	80.60	0.00
Phytase 3A1	96.06	0.51	0.75	0.76		72.40	
Phytase 3A2	96.02	0.50	0.74	0.75		71.60	
Phytase 3B	94.79	0.55	0.80	0.82		76.70	
Phytase 4D1	94.26	0.62	0.89	0.93		85.00	
Phytase 4D2	94.67	0.57	0.82	0.85		78.80	
Phytase TD1	98.07	0.88	1.26	1.37		119.00	
Phytase TD2	97.62	0.65	0.93	0.98		88.60	
3SC-22	94.3	0.53	0.77	0.79		74.2	
4SD-22	94.2	0.61	0.88	0.92		84.3	
TD1-22	**98.69**	**0.52**	**0.76**	**0.77**		**73.1**	
3SC-23	94.59	0.54	0.78	0.8		75.1	
3SF-23	93.9	0.57	0.82	0.85		79	
4SB-23	93.49	0.59	0.85	0.88		81.2	
4SA-23	94.35	0.61	0.88	0.92		84.3	
TD3-23	97.67	0.66	0.96	1.01		91	
TD2-23	97.93	0.65	0.94	0.99		89.7	
3SB-24	96.06	0.56	0.81	0.83		77.4	
3SA-24	95.54	0.48	0.73	0.74		70.5	
4SD-24	94.27	0.55	0.8	0.82		76.7	
4SA-24	94.45	0.58	0.84	0.87		80.3	
TD2-24	97.72	0.43	0.69	0.69		67	
TD1-24	98	0.51	0.75	0.76		72.3	
3SF-25	94.71	0.55	0.79	0.82		76.4	
4SD-25	94.41	0.54	0.78	0.80		74.90	
TD-25	98.59	0.41	0.68	0.68		66.00	

Yeast Extract Specification Sheet (referenced in Section 3.6.1.3)

Typical Amino Acid profile of Exl Prime LS

Amino Acid	%
Lysine	*3.84%*
Alanine	*3.32%*
Arginine	*2.66%*
Aspartic Acid	*5.01%*
Cystine	*0.72%*
Glutamic Acid	*8.89%*
Glycine	*2.37%*
Histidine	*0.95%*
Isoleucine	*2.24%*
Leucine	*3.19%*
Methionine	*0.82%*
Phenylalanine	*1.78%*
Proline	*2.03%*
Threonine	*2.11%*
Serine	*2.12%*
Tyrosine	*1.69%*
Valine	*2.93%*
Tryptophan	*0.59%*

Appendix E

Appendix E Statistical analyses

Cone and quartering analysis (referenced in section 3.3.1)

	Duplicate			
Analysis	1	2	Average	SS Dup
1	311	299	305.0	72.00
2	306	280	293.0	338.00
3	281	277	279.0	8.00
4	269	284	276.5	112.50
5	305	294	299.5	60.50
6	258	306	282.0	1152.00
7	277	301	289.0	288.00
8	305	335	320.0	450.00
9	289	288	288.5	0.50
10	311	301	306.0	50.00
			293.85	**2531.50**

Corrected SS	
294.1225	26.5225
147.6225	191.8225
165.1225	283.9225
617.5225	97.0225
124.3225	0.0225
1285.2225	147.6225
283.9225	51.1225
124.3225	1693.3225
23.5225	34.2225
294.1225	51.1225
TOTAL	**5936.55**

Source	df	SS	MS	F-ratio
Analysis	*9*	**3405.05**	**378.34**	**1.49**
Duplicate	*10*	**2531.50**	**253.15**	
TOTAL	*19*	**5936.55**		

Side activities (referenced in Section 3.3.5)

SUMMARY OUTPUT Side activities

Side Activities Analysis

Regression Statistics

Multiple R	0.168229
R Square	0.028301
Adjusted R Square	0.017259
Standard Error	51.29849
Observations	90

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	6744.704	6744.704	2.56303	0.112972733
Residual	88	231575.1	2631.535		
Total	89	238319.8			

Side activities continued (referenced in Section 3.3.5)

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	370.0954	25.75642	14.36905	8.37E-25	318.9098966	421.2809234	318.9098966	421.2809234
X Variable 1	0.512054	0.319844	1.600947	0.112973	-0.123570176	1.14767774	-0.123570176	1.14767774

	Phytase
Protease	-0.0593
Amylase	0.0296
Cellulase	0.1682
Xylanase	-0.0815

Level of Significance 0.113

all others are even less significant

Phytase activity and laboratory sample storage conditions – Data and Analysis (as referenced in Section 3.3.2)

Wet samples initial activity - immediate analysis				Wet samples kept at 4°C x 24 hours				wet samples stored at 21° C for 24 h			
Sample No.	*Activity*	*Activity*	*Moisture*	*Sample No.*	*Activity*	*Activity*	*Moisture*	*Sample No.*	*Activity*	*Activity*	*Moisture*
1	125	136	54.11%	*1*	129	125	54.23%	*1*	117	117	55.55%
	119	126	54.11%		121	111	54.23%		127	125	55.55%
2	115	107	54.11%	*2*	109	107	54.23%	*2*	130	131	55.55%
	117	116	54.11%		125	115	54.23%		131	135	55.55%
3	123	129	54.11%	*3*	111	116	54.23%	*3*	117	117	55.55%
	130	118	54.11%		104	114	54.23%		121	130	55.55%
4	118	118	54.11%	*4*	110	107	54.23%	*4*	125	132	55.55%
	115	109	54.11%		109	111	54.23%		120	132	55.55%
5	116	115	54.11%	*5*	117	117	54.23%	*5*	105	109	55.55%
	108	104	54.11%		106	105	54.23%		111	117	55.55%
6	107	112	54.11%	*6*	102	100	54.23%	*6*	113	119	55.55%
	119	111	54.11%		114	114	54.23%		113	115	55.55%

Phytase activity and laboratory sample storage conditions – Data and Analysis (as referenced in Section 3.3.2)

Wet samples initial activity - immediate analysis			
Sample No.	Activity	Activity	Moisture
1	125	136	54.11%
	119	126	54.11%
2	115	107	54.11%
	117	116	54.11%
3	123	129	54.11%
	130	118	54.11%
4	118	118	54.11%
	115	109	54.11%
5	116	115	54.11%
	108	104	54.11%
6	107	112	54.11%
	119	111	54.11%

Wet samples kept at 4°C x 24 hours			
Sample No.	Activity	Activity	Moisture
1	129	125	54.23%
	121	111	54.23%
2	109	107	54.23%
	125	115	54.23%
3	111	116	54.23%
	104	114	54.23%
4	110	107	54.23%
	109	111	54.23%
5	117	117	54.23%
	106	105	54.23%
6	102	100	54.23%
	114	114	54.23%

Phytase activity and laboratory sample storage conditions – Data and Analysis (as referenced in Section 3.3.2)

wet samples stored at 21° C for 24 h			
Sample No.	Activity	Activity	Moisture
1	117	117	55.55%
	127	125	55.55%
2	130	131	55.55%
	131	135	55.55%
3	117	117	55.55%
	121	130	55.55%
4	125	132	55.55%
	120	132	55.55%
5	105	109	55.55%
	111	117	55.55%
6	113	119	55.55%
	113	115	55.55%

Phytase activity and laboratory sample storage conditions – Data and Analysis (as referenced in Section 3.3.2)

Statistical analysis of Data

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.66749
R Square	0.44555
Adjusted R Squa	0.38490
Standard Error	6.74334
Observations	72

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	7	2338.63	334.09	7.35	0.0000
Residual	64	2910.25	45.47		
Total	71	5248.88			

	Coefficients	*standard Err*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	123.42	2.25	54.91	0.0000	118.93	127.91
Sample 2	-3.33	2.75	-1.21	0.2304	-8.83	2.17
Sample 3	-4.00	2.75	-1.45	0.1511	-9.50	1.50
Sample 4	-6.00	2.75	-2.18	0.0330	-11.50	-0.50
Sample 5	-12.33	2.75	-4.48	0.0000	-17.83	-6.83
Sample 6	-11.58	2.75	-4.21	0.0001	-17.08	-6.08
4 C for 24 hrs	-4.75	1.95	-2.44	0.0175	-8.64	-0.86
21 C for 24 hrs	4.00	1.95	2.05	0.0440	0.11	7.89

Phytase activity and laboratory sample storage conditions – Data and Analysis (as referenced in Section 3.3.2)

SUMMARY OUTPUT – Regression

Regression Statistics	
Multiple R	0.66749
R Square	0.44555
Adjusted R Square	0.38490
Standard Error	6.74334
Observations	72

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	7	2338.63	334.09	7.35	0.0000
Residual	64	2910.25	45.47		
Total	71	5248.88			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	123.42	2.25	54.91	0.0000	118.93	127.91
Sample 2	-3.33	2.75	-1.21	0.2304	-8.83	2.17
Sample 3	-4.00	2.75	-1.45	0.1511	-9.50	1.50
Sample 4	-6.00	2.75	-2.18	0.0330	-11.50	-0.50
Sample 5	-12.33	2.75	-4.48	0.0000	-17.83	-6.83
Sample 6	-11.58	2.75	-4.21	0.0001	-17.08	-6.08
4 C for 24 hrs	-4.75	1.95	-2.44	0.0175	-8.64	-0.86
21 C for 24 hrs	4.00	1.95	2.05	0.0440	0.11	7.89

Appendix E Pilot Dryer Experimentation - Statistical analysis for data from section 3.3.4

	pilot dryer	*All numbers average of two analyses*
	244	8.79
STD DEV	Average Phytase 19.89	moisture % 4.30
AV	196 **222**	17.38 **11.03**
	264	5.95
	220	10.29
	181	24.95
	204	13.36
	204	9.03
STD DEV	28.78	6.83
AV	**185**	**12.54**

wet samples before drying

76	55.01
91	55.71
89	54.51
87	56.87
97	56.54
103	55.55
STD DEV 9.40	0.89
AV **79**	**47.87**

samples after 4 h of drying at 70°C

221	34.00
183	35.53
153	38.02
193	36.19
174	35.78
218	37.98
309	30.70
256	28.50
206	27.63
197	33.63
269	25.90
302	29.22
STD DEV 50	4.20
AV **210**	**30.56**

samples after 8 h of drying at 70°C

252	11.41
224	13.81
246	17.69
230	14.41
258	12.90
220	18.75
198	6.54
262	10.30
242	4.25
224	12.04
264	8.18

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Glossary

Chapter 7: Glossary

Accuracy: Conformity of an indicated value to an accepted standard.

Alpha amylase: An enzyme that degrades starch, first to oligosaccharides and then in turn to maltose and glucose. (alpha 1,4-glucan 4-glucanohydrolase, EC 3.2.1.1).

ANF: Anti-nutritional factor (Phytate is an example of an anti-nutritional factor since it is a compound that interferes with nutrient absorption).

ANOVA: Analysis of Variance.

β-glucanase: Breaks down beta-linked glucose bonds (often associated with grains).

Cellulase: Cellulase refers to a group of enzymes which, acting together, hydrolyse cellulose.

Central composite design: An experimental design, useful in response surface methodology, for building a second order (quadratic) model.

Central Composite Face-Centred Design (CCFD): Requires only three levels for each factor and leads to a quadratic model.

CFU: Colony Forming Units.

CIP: Cleaning in Place.

CPH: Changes per Hour.

CMA Standard Agar: Corn Meal Standard Agar.

Cone and Quartering: A method for extracting sub-samples.

Conidia: Asexual spores.

Conidiophores: Fruiting structures, sporangia.

DNS: Dinitrosalicylic acid solution.

FAN: Free Amino Nitrogen.

FBD: Fluid Bed Drier.

HEPA: Filter that removes at least 99.97% of airborne.

Koji: Traditionally a `starter' consisting of Aspergillus cultured on a substrate.

Latin Square Design: The Latin square design is used where the researcher desires to control the variation in an experiment that is related to rows and columns in the field.

Minitab: A computer program designed to perform statistical functions.

Miso: A traditional Japanese food produced by fermenting rice, barley and/or soybeans, with salt and the mold kōjikin.

Monilia cinerea var Americana: A red fungus.

Natto: Natto is fermented soybeans.

Nested design: Takes the form of a tree-like structure.

NSP: Non-Starch Polysaccharides.

Oligotrophy: Capable of growing in nutrient-depleted environments.

PDA medium: Potato Dextrose Medium.

Phytase: An enzyme in plants that catalyses the breakdown (hydrolysis) of phytic acid into inositol and phosphoric acid.

Phytate: The salt form of phytic acid.

Phytic acid: Phytic acid (known as inositol hexakisphosphate (IP6), or phytate when in salt form) is the principal storage form of phosphorus in many plant tissues.

PLC: Programmable Logic Controller.

Protease: Proteinase that catalyses the splitting of interior peptide bonds in a protein.

Quadratic Equation: Equation where the highest power of x is x^2.

Reproducibility: Closeness of agreement among repeated measurements under the same operating conditions over time.

Reproducibility: The closeness of agreement among repeated measurements under the same operating conditions.

Response Surface Methodology: Used to achieve the optimisation of the experimental conditions.

Response Surface Models: May involve just main effects and interactions or they may also have quadratic and possibly cubic terms to account for curvature.

Rhizoids: Root-like hyphae.

RPM: Revolutions Per Minute, a unit of frequency.

RTD: Resistance Temperature Detectors.

Saké: A Japanese alcoholic beverage made from rice.

Serdán: Small city in Mexico where the Alltech SSF plant is located.

SMF: Submerged Fermentation.

Sporangiospore: A spore contained in a sporangium.

SSF: Solid- State Fermentation.

Definition: Solid- state fermentation (SSF) is defined as a fermentation involving solids in the absence (or near absence) of free water.

Soy sauce: A fermented sauce made from soybeans (soya beans), roasted grain, water and salt.

Std dev: A mathematical way to express how tightly a set of data tends to cluster around an average.

Takadiastase: Takadiastase is a form of diastase, which results from the growth, development, and nutrition of a distinct microscopic fungus known as *Aspergillus oryzae.*

Tempeh: A natural culturing and controlled fermentation process that binds soybean particles into a cake form.

TF: Tray filling.

Water activity: Water activity or Aw is the energy state of water in a substance.

Xylanase: Xylanase (EC 3.2.1.8) is the name given to a class of enzymes, which degrade the linear polysaccharide beta-1,4-xylan into xylose.